Nitric oxide for the prevention and treatment of viral, bacterial, protozoal and fungal infections [version 2; peer review: 1 approved, 2 approved with reservations]

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Abstract

Although the antimicrobial potential of nitric oxide (NO) is widely published, it is little used clinically. NO is a key signalling molecule modulating vascular, neuronal, inflammatory and immune responses. Endogenous antimicrobial activity is largely mediated by high local NO concentrations produced by cellular inducible nitric oxide synthase, and by derivative reactive nitrogen oxide species including peroxynitrite and S-nitrosothiols. NO may be taken as dietary substrate (inorganic nitrate, L-arginine), and therapeutically as gaseous NO, and transdermal, sublingual, oral, intranasal and intravenous nitrite or nitrate. Numerous preclinical studies have demonstrated that NO has generic static and cidal activities against viruses (including β-coronaviruses such as SARS-CoV-2), bacteria, protozoa and fungi/yeasts in vitro. Therapeutic effects have been seen in animal models in vivo, and phase II trials have demonstrated that NO donors can reduce microbial infection. Nevertheless, excess NO, as occurs in septic shock, is associated with increased morbidity and mortality. In view of the dose-dependent positive and negative effects of NO, safety and efficacy trials of NO and its donors are needed for assessing their role in the prevention and treatment of infections. Trials should test dietary inorganic nitrate for pre- or post-exposure prophylaxis and gaseous NO or oral, topical or intravenous nitrite and nitrate for treatment of mild-to-severe infections, including due to SARS-CoV-2 (COVID-19). This review summarises the evidence base from in vitro, in vivo and early phase clinical studies of NO activity.
in viral, bacterial, protozoal and fungal infections.

**Keywords**
Bacteria, COVID-19, fungus, nitric oxide, nitrate, nitrite, protozoa, virus

This article is included in the Pathogens gateway.
Introduction

Nitric oxide (NO), an inorganic molecule, is generated endogenously by prokaryotes and eukaryotes from L-arginine and L-citrulline by a family of NO synthase enzymes (NOS; Table 1.1, Table 1.2). In higher animals, it is also generated by reduction of dietary and endogenous nitrate (NO$_3^-$) to nitrite (NO$_2^-$) and thence NO (Table 1.3). NO is a pleiotropic signalling molecule involved in vascular, neuronal and metabolic regulation and has multiple physiological effects including lowering blood pressure, increasing exercise performance, and reversing metabolic syndrome. Underlying these processes, NO modulates multiple cell types including leucocytes, platelets, endothelial cells and smooth muscle cells, and neuronal, cardiac and renal function. Three isoforms of NOS exist in eukaryotes: neuronal (nNOS, NOS1), inducible (iNOS, NOS2) and endothelial (eNOS, NOS3). In multicellular organisms, NOS1-3 produces NO that broadly mediates neurotransmission, cyto-toxicity and vascular regulation respectively. Within cells, NO interacts with mitochondrial respiration, activates metabolic regulatory pathways and reduces oxidative stress.

As people age, endothelial-derived vascular NO levels fall and so vascular function declines causing relative endothelial dysfunction, pro-platelet and pro-inflammatory effects, and increased smooth muscle proliferation. Vascular NO levels are even lower in people with established vascular disease, e.g. those with stroke. Numerous viruses (including adenovirus, Coxsackie, coronavirus, cytomegalovirus, echovirus, herpes simplex, human T-cell leukaemia virus type-1, human immunodeficiency virus, influenza, measles, mumps, polio) and bacteria (Leptospira spp.) can infect and damage endothelium and so further reduce vascular NO levels. This also appears to occur in SARS-CoV-2 infection.

Most physiological effects of NO are modulated by cyclic guanosine monophosphate (cGMP, second messenger), and terminated when cGMP is metabolised by phosphodiesterase-5 (PDE5). This combined L-arginine/nitrate-NO-cGMP-PDE5 system (or nitric oxide system) comprises one of two key vasculo-protective pathways, the other being the prostaglandin-cyclic adenosine monophosphate-phosphodiesterase-3 pathway (PG-cAMP-PDE3, or prostacyclin system, see below).

The NO system may be enhanced or stimulated exogenously with substrate (L-arginine, L-citrulline, organic nitrate, inorganic nitrite or nitrate), NO gas, and PDE5 inhibitors. These can be inhaled or administered via transdermal, sublingual, oral, intranasal or intravenous routes (see below). Since endogenous NO generated by inducible NOS plays a key role in defence against multiple microbial pathogens (including viruses, bacteria, protozoa and fungi/yeast), this raises the possibility that exogenous NO might have therapeutic potential as a broad-spectrum antimicrobial, and this is the topic of this review.

Methods for the review

There are numerous publications in this research area and our intention was not to perform a systematic review of these; rather we present exemplars from the research field. We identified publications relating to the effect of NO on viruses, bacteria, protozoa and fungi/yeasts from searches of our own reference libraries, PubMed and Google, and reference lists given in earlier reviews and commentaries.

The primary searches for relevant studies for inclusion were done through PubMed from inception to 4 May 2021, in English with the following disease terms: “microbe” or “virus” or “bacteria” or “protozoa” or “fungi” or “yeast”. The results of these searches were crossed with the drug terms “nitric oxide” or “nitrite” or “nitrate”, and the identified abstracts were screened by one or more researchers. Earlier studies, including published reviews, were also identified from the files of the senior author. Studies included in vitro, in vivo and clinical trials. Although other drug classes, such as statins and angiotensin-converting enzyme inhibitors, enhance endothelial production of nitric oxide, their main effects are mediated through other pathways, and we have not assessed them here even though they may attenuate microbial disease severity.
Chemistry and biology of nitric oxide

NO is a small diatomic hydrophobic colourless gas that diffuses easily and has a short half-life. With an unpaired electron (NO), it is a free radical and so is chemically reactive and unstable in the presence of oxygen and superoxide producing reactive nitrogen oxide species (RNOS). In reality, the chemistry of NO is more complex and it exists in several redox forms: nitrosonium cation (NO⁺), NO (NO) and nitroxyl anion NO/C0).23

As an inorganic molecule, its central role in biology as a signalling molecule was only discovered in the 1980s,24 the identification of which led to the Nobel Prize for Physiology and Medicine in 1998.25 Ironically, the medicinal use of NO in the form of glyceryl trinitrate (GTN) for angina prophylaxis antedates the modern understanding of the biological synthesis and role of NO by more than a century.26

Experimental studies demonstrating nitric oxide inhibition of ...

Vira

Numerous preclinical in vitro studies have demonstrated that NO sources (stimulated endogenous NO, inorganic and organic nitrates, L-arginine) and PDE5-inhibitors can reduce replication in all seven types of virus as defined in the Baltimore classification (Table 2);27 this includes Class IV viruses (positive-sense RNA viruses) incorporating several coronaviruses28–33 including SARS-CoV-2.34–36 Most studies showed efficacy although neutral studies were reported for porcine reproductive and respiratory virus (an arterivirus, which is closely related to coronaviruses) and rhinovirus.37,38

Bacteria

Multiple studies have assessed the effect of NO on bacteria and inhibitory effects have been seen across a wide range of gram negative, gram positive and acid-fast bacteria (Table 3). NO sources included L-arginine, NO, nitrite, organic nitrates, and endogenously-generated NO. Multiple mechanisms for efficacy have been reported, as discussed below.

Table 1. Chemical equations relevant to the nitric oxide system.

| No. | Substrate | Product | Enzyme | Biochemical equation |
|-----|-----------|---------|--------|----------------------|
| 1.  | Dietary L-arginine | NO | Nitric oxide synthase | 2 L-arginine + 3 NADPH + 3 H⁺ + 4 O₂ → 2 L-citrulline + 2 NO + 3 NADP⁺ + 4 H₂O |
| 2.  | Dietary L-citrulline | L-arginine | Argininosuccinate synthetase, argininosuccinate lyase | L-citrulline + L-aspartate → argininosuccinate → L-arginine + fumarate |
| 3.  | Dietary nitrate | NO |  | NO₃⁻ + e⁻ + 2 H⁺ → H₂O + NO₂⁻ + 2 NO₂⁻ + 2 H⁺ → 2 HNO₂ → N₂O₃ + H₂O |
| 4.  | NO | Peroxynitrite |  | NO⁺ + O₂⁻ → ONOO⁻ |
| 5.  | Superoxide | Hydrogen peroxide | Superoxide dismutase | O₂⁻ → O₂ + H₂O |
| 6.  | Nitrite | Peroxynitrous acid |  | NO₂⁻ + H₂O₂ → ONOOH |
| 7.  | NO | Nitrogen dioxide |  | 2 NO + O₂ → 2 NO₂; ONOOH → NO₂ + OH |
| 8.  | NO | Dinitrogen trioxide |  | NO + NO₂ → N₂O₃ |
| 9.  | Nitrogen dioxide | Dinitrogen tetroxide (amyl) |  | 2 NO₂ → N₂O₄ |
| 10. | Hydrogen peroxide | Hydroxyl radicals |  | H₂O₂ → OH⁻ + OH⁺ → DNA damage |
| 11. | NO | Nitrous oxide | NO reductase | 2 NO + NAD(P) H + H⁺ → N₂O + H₂O + NAD(P)⁺ |
| 12. | NO | Nitrate | NO dioxygenase | Fe³⁺(O₂⁻) + NO → Fe⁵⁺ + NO₄⁻ |

Note: Many of these reactions can occur in the opposite direction, e.g. inhaled NO₂, an environmental pollutant, is a source of bioactive intravascular nitrite.195

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| Year | Virus (Baltimore class) | Disease (human unless stated) | NO source | In vitro cell lines | In vivo | Results |
|------|-------------------------|-------------------------------|-----------|---------------------|---------|---------|
| 2020 | Cytomegalovirus          | In compromised immune systems | DETA/NO   | MRC-5, ARPE-19      |         | Reduced replication |
| 1993 | Ectromelia              | Mousepox                     | SNAP      | 293                 | C57BL/6NCR mice | Reduced replication |
| 1994 | Epstein-Barr            | Infectious mononucleosis, Burkitt lymphoma | Constitutive lymphocyte | Human B-lymphocytes |         | Reduced reactivation |
| 1993 | Herpes simplex-1        | Cold sores                   | SNAP      | 293                 | C57BL/6NCR mice | Reduced replication |
| 2015 | Human papilloma virus   | Anogenital warts              | Acidified NaNO₂ |         | Human | Cure in 31% (active) vs 14% (control) |
| 1999 | Molluscipoxirus         | Molluscum contagiosum        | Acidified NaNO₂ |         | Human | Cure in 75% (active) vs 21% (control) |
| 1993 | Vaccinia                | Human “pox”                  | SNAP      | 293                 | C57BL/6NCR mice | Reduced replication |
| 1995 | Vaccinia                | Human “pox”                  | L-arg     | RAW 264.7           |         | Reduced replication |
| 1995 | Vaccinia                | Human “pox”                  | iNOS      | BSC-40, HeLa G      |         | Reduced replication |
| 2017 | Porcine circovirus-2   | Swine multisystemic wasting syndrome | GSNO      | PK-15               | BALB/c mice | Reduced replication & infection |
| 2009 | Porcine parvovirus      | Swine embryonic/foetal death  | SNAP, L-arg | PK-15               |         | Reduced replication |
| 1996 | Avian (ortho-) reovirus | Avian arthritis, tenosynovitis | LPS-stimulated macrophages | HD11 |         | Reduced replication |
| 1997 | Coxsackievirus (B3)     | Pleurodynia, myocarditis, pericarditis, and hepatitis | INOS transfection, SNAP | HeLa |         | Reduced replication, RNA and protein synthesis |
| 2006 | Dengue virus-2          | Viral haemorrhagic fever     | SNAP      | LLC-MK2 monkey kidney |         | Reduced RNA and protein synthesis |
| 1999 | Human immunodeficiency virus | Acquired immune deficiency syndrome | SNAP | Human monocytes, U1 cells |         | Reduced replication in monocytes. Increased replication in U1 cells |
| Year | Virus (Baltimore class) | Disease (human unless stated) | NO source | In vitro cell lines | In vivo results |
|------|-------------------------|-------------------------------|-----------|-------------------|----------------|
| 1997 | Japanese encephalitis virus | Encephalitis | SNAP, IFN-γ, activated macrophages | Murine RAW 264.7 and N18 cells | Reduced replication |
| 2005 | Mengovirus | Acute fever | Dipyridamole | HeLa or L cells | Reduced replication |
| 1997 | Murine coronavirus | Acute respiratory distress syndrome (ARDS) | NO gas | N18 cells and Hela cells | Reduced replication |
| 1998 | Poliovirus | Poliomyelitis | SNAP | HeLa, U937 | Reduced replication |
| 2010 | Porcine reproductive & respiratory virus | Swine respiratory, reproductive failure | SNAP, NAP | Marc-145 cells | Reduced replication |
| 2010 | Porcine respiratory coronavirus | Swine respiratory, reproductive failure | SNAP, NAP | Marc-145 cells | Reduced replication |
| 2020 | SARS-CoV-2 | COVID-19 | Dipyridamole | Human (n=31) | Reduced replication |
| 2020 | SARS-CoV-2 | COVID-19 | SNAP, SNP, SIN-1 | Vero E6 | Reduced replication, recombinant protease activity |
| 2020 | SARS-CoV-2 | COVID-19 | SNK | Vero E6 | Reduced replication, recombinant protease activity |

**Table 2. Continued**
| Year | Virus (Baltimore class) | Disease (human unless stated) | NO source | In vitro cell lines | In vivo | Results |
|------|-------------------------|-------------------------------|-----------|---------------------|---------|---------|
| 2006 | (Ortho)hantavirus       | Haemorrhagic fever/pulmonary syndrome | SNAP, SIN-1 | Vero E6 | C57BL/6 (iNOS<sup>−/−</sup>, +/+<sup>+</sup>) mice | Reduced replication |
| 2006 | Parainfluenza virus     | 'Cold'                         | DetaNONOate, SNAP, INOS overexpression | Cystic fibrosis epithelial cells | | Reduced replication |
| 2001 | Rabies virus            | Rabies                         | SNP + ascorbate | Neuroblastoma cells | | Reduced replication |
| 1995 | Vesicular stomatitis    | 'Flu. Bovine oral ulcers       | SNAP       | NB41A3              | | Reduced replication |
| 1995 | Friend leukaemia + spleen focus-forming | Murine leukaemia | SIN-1, SNP, SNAP | Dunni | | Reduced replication (but not with NaNO<sub>2</sub>) |
| 2000 | Hepatitis B             | Viral hepatitis                | IFN-γ      | HBV transgenic/INOS knockout mice | NO mediates antiviral activity of IFN-γ |

DETA/NONOate: diethylenetriamine NONOate; dsDNA: double-strand DNA (type I); dsDNA-RT: single-strand DNA-retro (type VII); dsRNA: double-strand RNA (type II); GSNO: S-nitrosoglutathione; IFN-γ: interferon-gamma; iNOS: inducible nitric oxide synthase; L-arg: L-arginine; Lϕ: lymphocyte; NaNO<sub>2</sub>: sodium nitrite; NAP: N-acetylpenicillamine; NO: nitric oxide; SARS: severe acute respiratory syndrome; SIN-1: 3-morpholinosydnoneimine; SNAP: S-nitroso-L-acetylpenicillamine; SNP: sodium nitroprusside; ssDNA: single-strand DNA (type II); (-)ssRNA: negative-sense single-strand RNA (type V); (+)ssRNA: positive-sense single-strand RNA (type IV); ssRNA-RT: single-strand RNA-retro (type VI).
| Year | Bacteria                  | Disease (human unless stated)          | NO source                                                                 | In vitro                                                                 | In vivo                                                                 | Results                                                                 |
|------|---------------------------|----------------------------------------|---------------------------------------------------------------------------|--------------------------------------------------------------------------|------------------------------------------------------------------------|-------------------------------------------------------------------------|
| 2005 | Acinetobacter baumanii    | ICU organisms                          | NO gas (200 ppm)                                                          | Bacterial cfu                                                           | No viable bacteria by 4.8 (±1.3) hr                                     |                                                                         |
| 2005 | Enterobacter aerogenes     | ICU organisms                          | NO gas (200 ppm)                                                          | Bacterial cfu                                                           | No viable bacteria by 4.8 (±1.3) hr                                     |                                                                         |
| 1993 | Brucella abortus          | Brucellosis                             | Activated macrophages (IFN-γ)                                             | Reduced cfu                                                              | Reduced cell viability                                                 |                                                                         |
| 1993 | Burkholderia pseudomallei | Meningitis acute                        | Activated macrophages (IFN-γ)                                             | Reduced cfu                                                              | Reduced cell viability                                                 |                                                                         |
| 1992 | Chlamydia trachomatis     | Trachoma, pelvic inflammatory disease   | Activated McCoy cells (IFN-γ)                                             | Reduced infectivity                                                     |                                                                         |                                                                         |
| 1995 | Ehrlichia                 | Ehrlichiosis                            | Activated macrophages (IFN-γ)                                             | Reduced cell viability                                                  |                                                                         |                                                                         |
| 1995 | Salmonella enterica       | Typhoid fever                           | Activated macrophages (IFN-γ)                                             | Reduced infection                                                       |                                                                         |                                                                         |
| 1992 | Francisella tularensis    | Tularemia                               | Activated macrophages (IFN-γ)                                             | Reduced cell viability                                                  |                                                                         |                                                                         |
| 1992 | Helicobacter pylori       | Gastritis, gastric duodenal ulcers     | Activated macrophages (IFN-γ)                                             | Reduced cell viability                                                  |                                                                         |                                                                         |
| 1992 | Legionella                | Legionnaires/ Pontiac fever             | Activated macrophages (IFN-γ)                                             | Reduced cell viability                                                  |                                                                         |                                                                         |
| 1992 | Pseudomonas aeruginosa    | Typhoid fever                           | Activated macrophages (IFN-γ)                                             | Reduced cell viability                                                  |                                                                         |                                                                         |
| 1992 | Rickettsia                | Spotted fever, typhus                   | Activated macrophages (IFN-γ)                                             | Reduced cell viability                                                  |                                                                         |                                                                         |
| 1995 | Salmonella enterica       | Typhoid fever                           | Activated macrophages (IFN-γ)                                             | Reduced cell viability                                                  |                                                                         |                                                                         |
| 2005 | Serratia marcescens       | ICU organisms                          | NO gas (200 ppm)                                                          | Bacterial cfu                                                           | No viable bacteria by 4.8 (±1.3) hr                                     |                                                                         |
| Year | Bacteria | Disease (human unless stated) | NO source | In vitro | In vivo |
|------|----------|--------------------------------|-----------|----------|---------|
| 2005 | *Stenotrophomonas maltophilia* | ICU organisms | NO gas (200 ppm) | Bacterial cfu | No viable bacteria by 4.8 (±1.3) hr. |
| 1992 | *Yersinia pestis* | Plague (bubonic, pneumonic, septicaemic) | NaNNO2, NaNNO3, KNO3 | Pork meat | Reduced growth |
| 1976 | *Bacillus cereus* | Gastroenteritis | Nitrosothiols (RSN=O) | Suspension of spores | Inhibition of spore germination |
| 1981 | *Clostridium perfringens* | Gastroenteritis | Sodium nitrite | Sublethal inoculum | Reduction of cfu, G AP-DH and aldolase activity, and free sulfhydryl groups |
| 1994 | *Listeria* | Listeriosis | L-NMMA | C57BL/6 mice | Reduced growth |
| 2005 | *Staphylococcus aureus* | ICU organisms | NO gas (200 ppm) | Bacterial cfu | No viable bacteria by 4.8 (±1.3) hr. |
| 2012 | *Staphylococcus aureus* | Wound infection | Probiotic NO gas patch | Ischaemic/injected S. aureus wounds in rabbits | Ischaemic/injected S. aureus wounds in rabbits |
| 2013 | *Staphylococcus aureus* | Experimental biofilms | Biofilm | Isosorbide mononitrate | Increased dispersal (conversion of sessile to planktonic cells) |
| 2005 | *Streptococci (group B)* | ICU organisms | NO gas (200 ppm) | Bacterial cfu | No viable bacteria by 4.8 (±1.3) hr. |
| 1991 | *Mycobacterium avium* | Atypical respiratory TB | Activated macrophages (TNF) | Human macrophages | Smear positive TB |
| 1991 | *Mycobacterium leprae* | Leprosy | Activated M. leprae metabolism | Activated macrophages (IFN-γ) | Reduced M. leprae metabolism |
| 2003 | *Mycobacterium ulcerans* | Buruli skin ulcer | Acidified nitrite (40 mM) | Buruli skin ulcer | Smear positive TB |
| 2004 | *Mycobacterium leprae* | Experimental biofilms | Activated macrophages (TNF) | Human macrophages | Smear positive TB |

cfu: colony forming units; GSNO: S-nitrosoglutathione; IFN-γ: interferon-gamma; MRA: methicillin resistant S. aureus; ppm: parts per million; SIN-1: 3-morpholinosydnonimine; TB: tuberculosis; TNF: tumour necrosis factor.
| Year | Protozoa             | Disease (human unless stated) | NO source | In vitro | In vivo |
|------|----------------------|------------------------------|-----------|----------|---------|
| 1992 | Entamoeba histolytica | Amoebiasis                   | Activated macrophages (IFN-γ/LPS) | Reduced infection | Destruction of amoebae |
| 1992 | Naegleria fowleri     | Meningitis                   | Activated macrophages (BCG)       | Reduced infection, and improved outcome | Larval cytotoxicity |
| 1994 | Opisthorchis felineae | Schistosomiasis, intestinal  | Activated macrophages (TNF)       | Reduced amastigotes and promastigotes | Reduced granuloma formation |
| 1989 | Schistosoma mansoni   | Schistosomiasis, intestinal  | Endogenous from iNOS              | Reduced amastigotes and promastigotes | Reduced intracellular parasites |
| 2000 | Leishmania major      | Leishmaniasis                | Activated macrophages (IFN-γ/LPS) | Reduced intracellular parasites | Reduced growth |
| 2000 | Leishmania major      | Leishmaniasis                | Activated macrophages (IFN-γ/LPS) | Reduced amastigotes and promastigotes | Reduced granuloma formation |
| 1989 | Trypanosoma brucei    | African trypanosomiasis      | Activated macrophages (IFN-γ/LPS) | Reduced amastigotes and promastigotes | Reduced granuloma formation |

BGC: bacillus Calmette-Guerin; GTN: glyceryl trinitrate; IFN-γ: interferon-gamma; iNOS: inducible nitric oxide synthase; LPS: lipopolysaccharide; SNAP: S-nitroso-L-acetylpenicillamine; SNP: sodium nitroprusside; TD: transdermal; TNF: tumour necrosis factor.
Table 5. Studies assessing the effect of nitric oxide on a non-inclusive list of fungi and yeasts.

| Year | Fungi/Yeasts | Disease (human unless stated) | NO source | In vitro | In vivo | Results |
|------|--------------|-------------------------------|-----------|----------|---------|---------|
| 1999 | *Aspergillus fumigatus* | Aspergillosis | Activated macro-phages (IFN-γ) | Rat alveolar macrophages | | Reduced infection |
| 1998 | *Epidermophyton floccosum* | Tinea pedis | Acidified NaNO₂ | | Human (n=35) | Cure in 81% (active) vs 31% (control) |
| 1994 | *Histoplasma capsulatum* | Histoplasmosis (‘flu-like) | IFN-γ/LPS activated macrophages | C57BL/6 mice | | Reduced infection |
| 1999 | *Pneumocystis carinii* | Pneumonia | IFN-γ activated macrophages via L-arginine | Sprague Dawley rats | | Killed P. carinii |
| 1998 | *Trichophyton rubrum, T. interdigitale* | Tinea pedis | Acidified NaNO₂ | Human (n=35) | | Cure in 81% (active) vs 31% (control) |
| Yeasts | 1993 | *Candida albicans* | Candidiasis (oropharyngeal, vulvovaginal, candidaemia) | Murine macrophages | C. albicans infection | Mice | Reduced infection |
| 1991 | *Cryptococcus neoformans* | Cryptococcosis (pneumonia, meningitis, encephalitis) | Acidified NaNO₂ | C. neoformans culture | | Reduced replication |
| 1999 | *Cryptococcus neoformans* | Cryptococcosis | Activated macro-phages (IFN-γ) | Rat alveolar macrophages | | Reduced infection |
| 2018 | Dermatophytes | Onychomycosis, tinea pedis | NVN1000 | | Macrodiution broth test | 78-99% kill |

IFN-γ: interferon-gamma; LPS: lipopolysaccharide; NaNO₂: sodium nitrite.
Protozoa
NO sources have been tested on both intracellular and extracellular protozoa (Table 4) with sources involving activated macrophages, sodium nitrite, glyceryl trinitrate, sodium nitroprusside (SNP) and S-nitroso-L-acetylpenicillamine (SNAP).

Fungi and yeasts
The effects of NO on several fungi and yeasts have been studied (Table 5). NO was donated exogenously through stimulating macrophages or as acidified nitrite. In vitro experiments demonstrated reduced replication whilst in vivo experiments in mice showed reduced infection.

Derivatives of nitric oxide
Whilst endogenous NO derived from eNOS and nNOS is physiologically active via its second messenger (cGMP), the antimicrobial effects of NO relate to its toxic effects when present at higher concentrations. Although it is technically challenging to measure free NO concentrations, studies suggest that NO concentrations derived from iNOS are 10–100× higher than those resulting from eNOS/nNOS (Table 6). NO concentrations resulting from exogenous administration lie between those from eNOS/nNOS and iNOS but approximate more closely to those from iNOS than eNOS. Importantly, much antimicrobial NO activity is likely to reflect the effects of derivative molecules rather than NO itself:

- Nitric oxide (NO). In general, bacteria deficient in low molecular weight thiols such as glutathione (e.g. Staphylococci spp.) are sensitive to attack by NO whereas those with high thiol concentrations are resistant to NO.
- Peroxynitrite (OONO-, Table 1.4). The reaction between NO and superoxide means that NO synergises with the respiratory burst, another antimicrobial system present in phagocytic cells. Experimentally, this synergism can be inhibited with the addition of superoxide dismutase which converts superoxide into molecular oxygen and hydrogen peroxide (Table 1.5).
- Peroxynitrous acid (ONOOH, Table 1.6), e.g. toxic to Escherichia coli.
- Nitrogen dioxide (NO2, Table 1.7), e.g. toxic to E. coli.
- Dinitrogen trioxide (N2O3, Table 1.8).
- Dinitrogen tetroxide (N2O4, Table 1.9).
- S-nitrosothiols (RSNO, e.g. S-nitrosoglutathione), e.g. toxic to E. coli and Salmonella enterica serovar typhimurium.40 RSNO reacts with protein thiol groups changing their function. Thiol concentrations do not appear to determine sensitivity to peroxynitrite and S-nitrosothiols.

Table 6. Nitric oxide concentrations following endogenous synthesis by nitric oxide synthase, and exogenous NO donors.

| NO       | eNOS/nNOS                     | iNOS                                      | Exogenous NO |
|----------|-------------------------------|-------------------------------------------|--------------|
| Source   | Endothelium, neurones         | Intracellular, e.g. macrophages           | Exogenous    |
| Role     | Cell signalling               | Microbial killing                         | Vasodilation, antiplatelet |
| Synthesis| Constitutive, intermittent    | Inducible, continuous (“fire hose”)15,    |              |
|          | (“dripping tap”15), calcium-dependent, feedback controlled | calcium-independent/cytokine-microbial dependent, Part of innate immunity |              |
| Concentration | 0.1-5 nM248                    | >10 μM248                                 | SNP, 52 nM249 |
| Targets  | sGC (CcOX)                    | Aconitase, NADH dehydrogenase, succinate dehydrogenase, metalloenzymes, ribonucleotide reductase, DNA | sGC |
| Effects  | Reversible                    | Irreversible. Nitrosation, nitration, oxidation. | Reversible   |

CcOX: cytochrome c oxidase (Complex IV); sGC: soluble guanylate cyclase; SNP: sodium nitroprusside.
Dinitrosyl-iron ((2 RS)-Fe-(2 NO)). The reaction of NO with iron or iron–sulphur molecules can: inactivate enzymes such as aconitase (which converts citrate to isocitrate in the citric acid cycle), ribonucleotide reductase and ubiquinone reductase; increase free ferrous (Fe^{2+}) which causes oxidative damage; and deplete iron stores.

RNOS (especially auto-oxidised products of NO).

Since these molecules differ in their stability, reactivity, location and cellular diffusivity, the overall effect of NO will depend on the molecular species involved and its location.

Cellular and other targets of nitric oxide

The targets for NO and associated reactive nitrogen species are multitudinous:

- DNA, through deamination of adenine, cytosine and guanine; cross-linking; breakage of strands; inhibition of DNA repair enzymes such as DNA alkyl transferases (and so preventing transfer of the guanine alkyl group to protein); and disruption of DNA replication by inhibition of ribonucleotide reductase; as in *S. enterica* and vaccinia virus.

- RNA, through disruption of RNA replication by inhibition of viral ribonucleotide reductase.

- Inhibition of mitochondrial function, specifically through inactivation of iron-sulphur complexes within respiratory chain enzymes.

- Protein modification at cysteine, methionine, phenylalanine, tryptophan and tyrosine residues, *e.g.* by RNOS. Such protein effects will reduce enzyme activity, as seen for DNA, proteases and mitochondrial function, as in Coxsackievirus.

- Limit late protein synthesis, *e.g.* through posttranslational modification of viral proteases. (Early protein translation/synthesis is not typically affected.)

- Induction of lipid peroxidation.

- Limit glutaminolysis by shuttling glutamine to glutathione synthesis, as in cytomegalovirus.

- Interaction with sulphydryl-containing constituents of the bacterial cell.

- Disrupt zinc homeostasis, as in *S. enterica*.

- Limit virion assembly/particle formation.

- Reduce bacterial adhesion to NO-releasing surfaces.

Nitric oxide may also play an augmenting role as an antimicrobial agent. Examples include the adjuvant roles of NO when given with type I interferons in the treatment of DNA viruses and L-arginine when given with conventional chemotherapy in smear-positive TB.

In addition, NO’s vasculo-active effects are likely to be beneficial in preventing infection and its severity, with NO:

- Reversing endothelial dysfunction and so potentially reducing endotheliitis, as occurs in COVID-19.

- Reducing leucocyte function (*e.g.* adhesion, chemotaxis, phagocytosis); COVID-19 is associated with increased phagocyte counts.

- Reducing platelet activation and platelet–leucocyte conjugation and so reducing micro- and macro-thrombosis, as seen in COVID-19.

- Improving organ blood flow and perfusion through smooth muscle relaxation and vasodilatation and so likely reversing infection-related vasoconstriction as seen in COVID-19, including in the pulmonary circulation.
These actions of NO are all mediated via the second messenger cGMP.

**Antimicrobial production of nitric oxide**

NO is produced by some bacteria, archaea and yeasts via several pathways including denitrification of nitrate to nitrite and then to NO \(^{54}\) and oxidation of L-arginine to NO and L-citrulline as catalysed by a bacterial nitric oxide synthase (bNOS), a process that can be inhibited by NOS inhibitors.\(^{55}\) Whilst eukaryotic NOS contains both catalytic and reductase domains, prokaryotic bNOS lacks the latter relying instead on other cellular reductases to generate NO; the one exception to this is the bNOS present in *Sorangium cellulosum* which does include a reductase module.

In contrast to the signalling role of NO in mammals, NO synthesis in bacteria has multiple functions which vary between antimicrobial species: \(^{56-59}\)

- Protection against oxidative stress with NO limiting thiol reduction and so the formation of hydroxyl radicals (*Bacillus anthracis/subtilis, Staphylococcus aureus*) \(^{56}\). (Table 1.10).

- Protection against oxidative stress with NO activating catalase (*B. subtilis*). Such defence will limit damage from phagocytic respiratory bursts.\(^ {56}\)

- Protection against oxidative stress by reducing endogenous NO synthesis and increasing the expression of NO dioxygenase to detoxify NO (*Candida albicans*).\(^ {58}\)

- Biosynthesis of toxins, e.g. thaxtomin (a phytotoxin) interfere with potato plant wall synthesis (*Streptomyces turgidiscabies*).\(^ {56}\)

- Activation of aerobic and nitrate respiration to optimise growth (*S. aureus*).\(^ {59,60}\)

- Protection against antimicrobial agents including amoxycillin, cefuroxime, gentamicin and novobiocin (*B. anthracis/cereus/thuringiensis/weihenstephanensis, S. aureus*), \(^ {57,58}\) and azoles (*Candida albicans*).\(^ {58}\)

The production by some microbes of endogenous NO to protect against oxidative stress is ironic since hosts are using NO to try to destroy the microbe!

**Resistance to nitric oxide**

Microbial resistance to antibiotics is an increasingly common problem and has left some bacteria with few treatment options, e.g. drug-resistant *Neisseria gonorrhoeae*. Hence, it is vital to consider whether resistance to NO is innate in some microbes or can be acquired. As already highlighted, some microbes have an intrinsic ability to produce their own NO and so attenuate the effects of oxidative stress (e.g. *B. anthracis/subtilis, C. albicans, S. aureus*),\(^ {56,59}\) activate aerobic respiration (*S. aureus*\(^ {59}\)) or protect against antimicrobial agents (*B. anthracis/cereus/thuringiensis/weihenstephanensis, C. albicans, S. aureus*\(^ {57,58}\)).

Microbes may also have mechanisms for deactivating NO. One mechanism is via a NO reductase which reduces NO to nitrous oxide and then nitrogen, e.g. as occurs in fungi\(^ {61}\) (Table 1.11). Bacteria have different NO reductases but similarly produce nitrous oxide,\(^ {61}\) as seen in *Pseudomonas aeruginosa*.\(^ {54}\) Loss-of-function mutations in NO reductase may be lethal, possibly because intracellular NO concentrations rise to toxic levels.

A second mechanism for detoxifying NO is via NO dioxygenase oxidation to nitrate (Table 1.12). The pre-eminent NO dioxygenase is flavohaemoglobin,\(^ {62}\) as present in bacteria (e.g. *Salmonella enterica, S. aureus, Vibrio cholerae, Yersinia pestis*),\(^ {52,63,64}\) and yeasts. A related haemoglobin, truncated haemoglobin, detoxifies NO in mycobacteria. Of note, *Mycobacterium leprae* has undergone reductive genome evolution losing more than 2,000 genes, including some that protect against RNOS; as a result, *M. leprae* has fewer defences against NO than *Mycobacterium tuberculosis*.\(^ {65}\) Bacterial lactate dehydrogenase also detoxifies NO, as seen in *S. aureus*.\(^ {57,58}\) Importantly, these detoxifying enzymes only cope with low levels of NO and are not protective against high NO levels.

As a result, microbes show differing sensitivities to NO, as seen for common airways pathogens where sensitivity was ranked (sensitivity most to least): *P. aeruginosa* ~ *C. albicans* > *S. aureus* > *Klebsiella pneumoniae* ~ *Staphylococcus epidermis*.\(^ {66}\)
However, there is little evidence that bacteria can acquire de novo resistance to NO, as confirmed in experiments on strains of *E. coli*, *P. aeruginosa*, *S. aureus* and *Staphylococcus epidermidis*. This property is unsurprising since NO has multiple mechanisms for antimicrobial activity and these are likely to be invoked orders of magnitude faster than any microbe can process metabolically, especially if protein synthesis is required. Equally, the main mechanisms for antibiotic resistance (drug inactivation, altered binding sites or metabolism and reduced drug permeability) are unlikely to be relevant to many NO sources. Whether viruses, protozoa and fungi can develop resistance to NO remains unclear.

**Administering nitric oxide, donors and related compounds**

### L-arginine and L-citrulline

In the presence of NOS, administration of L-arginine may enhance NO synthesis (Table 7) although intracellular L-arginine levels are not normally rate limiting and so administration may not have physiological effects. Although oral preparations of L-arginine are commercially available, consumption of high doses is associated with profuse diarrhoea (P Bath, personal observation). However, L-citrulline may be a more efficient method for delivering L-arginine since it has more efficient intestinal absorption, lower first pass metabolism and higher renal reabsorption, does not induce arginase and is safe and tolerable at high doses.

### Inhaled nitric oxide

Gaseous NO may be inhaled with the aim of improving pulmonary haemodynamics and killing microbes. Multiple trials are underway for COVID-19 prevention and treatment (Table 9). NO may also be created in real time by combining sodium nitrite and citric acid and administering this either as a nasal spray (for local therapy) or via nebuliser (for combined nasal and bronchial therapy).

### Organic nitrates

Organic nitrates such as GTN, isosorbide dinitrate (ISDN) and isosorbide mononitrate (ISMN) are widely used in vascular medicine for the prevention and treatment of angina, treatment of chest pain in unstable angina and myocardial infarction, treatment of severe heart failure, and blood pressure lowering after cardiac surgery and in acute stroke (Table 7). There is increasing concern that chronic use of organic nitrates may cause major adverse cardiac events and death, reduce daily activity, and not improve quality of life or exercise capacity. Potential explanations include the development of tolerance, and induction of endothelial dysfunction and cell damage through oxidative stress, *e.g.* production of free radicals/peroxynitrite. Importantly, organic nitrates only release NO in cells and tissues expressing mitochondrial aldehyde dehydrogenase-2. For example, SNP and SIN-1 inhibit monocyte chemotaxis whilst organic nitrates (ISDN, GTN and molsidomine) do not; this contrasts with smooth muscle cells which vasodilate with all five agents. Since aldehyde dehydrogenase-2 suffers from use-inactivation, nitrate tolerance (tachyphylaxis) and endothelial dysfunction develops and bioconversion only restarts following a nitrate-free period. Several *in vitro* studies have demonstrated the potential antimicrobial effects of organic nitrates (Table 7). Other non-organic nitrates include pentaerythritol tetranitrate and erythrityl tetranitrate.

### Therapeutic inorganic nitrite and nitrate

Therapeutic use of inorganic nitrite is limited with intravenous administration used in cyanide poisoning (British National Formulary). Topical acidified sodium nitrite has been shown to reduce cutaneous infections secondary to a variety of viruses and bacteria although its general use is probably limited by skin irritation and erythema (Table 7). A recent study found that acidified nitrite improved wound healing in rats with diabetes.

### Dietary inorganic nitrate

NO may also be produced from dietary inorganic nitrate, as is present in high concentrations in green leafy and some root vegetables, *e.g.* spinach, lettuce, rocket, beetroot, celery, fennel, radish and Chinese cabbage. Nitrate is absorbed from the proximal gastrointestinal tract, excreted by salivary glands, reduced to nitrite by oral bacteria and then absorbed in the gastrointestinal tract. A number of bacterial species situated on the dorsal surface of the tongue perform this conversion via nitrate reductases. In the absence of oxygen, nitrate and nitrite are commonly used by bacteria as terminal electron acceptors for respiration. Through this symbiotic relationship, the mammalian host provides the nutrients and the environment in return for nitrite production by bacteria.

Absorbed and circulating nitrite is then further reduced to NO, a process that is enhanced in hypoxic or acidic conditions and by multiple mechanisms including deoxyhaemoglobin, deoxymyoglobin, xanthine oxidoreductase and endothelial nitric oxide synthase. As such, most effects of dietary nitrate will be vascular and perivascular. The beneficial vascular protective effects of vegetable consumption are very clear epidemiologically, as present in the classical Japanese
Table 7. Nitric oxide sources.

| Intervention       | Example                                           | Administration | Licensed for use in (BNF) | Antimicrobial effects: target (disease)                                                                 |
|--------------------|---------------------------------------------------|----------------|---------------------------|----------------------------------------------------------------------------------------------------------|
| L-arginine         | Dietary: meat                                     | Oral           | N/A                       | None reported                                                                                           |
| Powder             |                                                   |                |                           | Mycobacteria tuberculosis (pulmonary tuberculosis)                                                     |
| Liquid             |                                                   | Intravenous    | N/A                       | None reported                                                                                           |
| L-citrulline       |                                                   | Oral           | N/A                       | None reported                                                                                           |
| Inorganic nitrite  | Acidified sodium nitrite (NaNO₃) cream            | Topical        |                           | Burkholderia cepacia, dermatophytes (tinea pedis), pox virus (molluscum contagiosum), Mycobacterium ulcerans (Buruli ulcer), human papilloma virus (anogenital warts), Propionibacterium acnes, P. aeruginosa, S. aureus (including MRSA) |
|                    |                                                   | Oral           | N/A                       | None reported                                                                                           |
|                    |                                                   | Intravenous    |                           | Cyanide poisoning (given with sodium thiosulfate)                                                      |
|                    |                                                   |                |                           | Food preservation: Clostridium botulinum                                                              |
| Inorganic nitrate  | Silver nitrate                                    | Topical        |                           | Human papilloma virus (Common wart)                                                                  |
|                    |                                                   |                |                           |                                                          |
| Organic nitrate    | Glyceryl trinitrate (GTN)                         | Topical patch  |                           | Prophylaxis of angina and phlebitis                                                                  |
|                    |                                                   | Ointment       |                           | Malaria (murine cerebral malaria), Non-specific infections (presenting as stroke mimics)            |
|                    |                                                   | Sublingual     |                           | None reported                                                                                           |
|                    |                                                   | Intravenous    |                           | Hypertension/myocardial ischaemia after cardiac surgery, Congestive heart failure, Unstable angina    |
|                    |                                                   |                |                           | E.coli, P. Aeruginosa, S. aureus in solution, S. aureus, MRSE, P. aeruginosa, C. albicans in biofilms |
|                    |                                                   | Oral           |                           | Prophylaxis/treatment of angina. Left ventricular failure                                            |
|                    |                                                   | Sublingual     |                           | None reported                                                                                           |
|                    |                                                   | Intravenous    |                           | None reported                                                                                           |
|                    |                                                   |                |                           |                                                          |
| Isosorbide dinitrate (ISDN) | | Oral           |                           | Prophylaxis/treatment of angina. Left ventricular failure                                            |
|                    |                                                   | Sublingual     |                           | None reported                                                                                           |
|                    |                                                   | Intravenous    |                           | None reported                                                                                           |
|                    |                                                   |                |                           |                                                          |
| Isosorbide mononitrate (ISMN) | | Oral           |                           | Prophylaxis of angina, Adjunct in congestive heart failure. S. aureus in biofilms                |

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| Intervention                        | Example                        | Administration | Licensed for use in (BNF)                                                                 | Antimicrobial effects: target (disease)                                                                 |
|------------------------------------|--------------------------------|----------------|------------------------------------------------------------------------------------------|----------------------------------------------------------------------------------------------------------|
| Spontaneous nitric oxide donors    | Sodium nitroprusside (SNP)    | Intravenous    | Hypertensive emergencies. Controlled hypotension. Acute/chronic heart failure              | *E. coli*, *P. Aeruginosa*, *S. aureus* in solution. *Bacillus licheniformis*, *Candida albicans*, *Escherichia coli* BW20767, *Fusobacterium nucleatum*, *Serratia marcescens* MG1, *S. epidermidis*, *Vibrio cholerae* 92A1552, *Leishmania* spp. |
| Nitric oxide                       | Nitric oxide (NO) gas         | Gas            | (Neonatal pulmonary hypertension)                                                          | *E. coli*, *P. aeruginosa*, *S. aureus*. SARS-CoV-2 (ongoing trials: NCT04290871, NCT04305457, NCT04312243). |
|                                    |                               | Probiotic patch | N/A                                                                                      | *E. coli*, *S. aureus*, MRSA, *P. aeruginosa*, *T. mentagrophytes*, *T. rubrum*. |
|                                    |                               | NO releasing solution | N/A (in development)                                                                    | *Propionibacterium acnes*, *T. mentagrophytes*, *T. rubrum*. SARS-CoV-2 (ongoing trial: NCT04337918) |
| PDE5 inhibitor                      | Dipyridamole                   | Oral           | Post-stroke prophylaxis.                                                                  | Picornaviridae, Togaviridae, Orthomyxoviridae, Paramyxoviridae, Herpetoviridae and Poxviridae. Mengovirus, SARS-CoV-2 (COVID-19 clinical trial). |
| Sildenafil                          | Oral                           | Oral           | Erectile dysfunction, pulmonary arterial hypertension                                       | Adenovirus, Chikungunya, Cytomegalovirus, Dengue, Enterovirus 71, Influenza virus, Measles, Mumps, Rabies, Respiratory syncytial virus, Rubella, West Nile, Yellow Fever; Methicillin-resistant *Staphylococcus epidermidis*. |

BNF: British National Formulary; N/A: not applicable.
diet, the Dietary Approaches to Stop Hypertension (DASH) diet, and the Mediterranean diet. Further, vegetable-derived nitrate may reduce the risk of gastrointestinal cancer. The benefit on cancer is at variance with oral consumption of nitrite. Although nitrite is not carcinogenic per se, the processing and cooking of nitrite-cured meat can form carcinogens such as N-nitroso compounds and heterocyclic aromatic amines. In contrast, carcinogens are not formed when eating raw vegetable-derived nitrate. A recent meta-analysis showed an increased risk gastric cancer with oral nitrite but reduced risk with oral nitrate. Dietary nitrate is known to modify the oral and gastric biome (Table 7).

High dietary intake of nitrate is associated with many mechanisms that may have beneficial vascular, and potentially, antimicrobial effects. Experimentally, beetroot juice is often used as a potent source of dietary nitrate since dosing can be controlled and a nitrate-free placebo version is available for use in randomised controlled trials. Studies have shown that beetroot juice increases plasma nitrate and nitrite concentrations, that most vascular effects are mediated via the second messenger cGMP, tolerance does not develop (unlike with organic nitrates) and inorganic nitrate does not lead to free radical formation. In clinical studies, beetroot juice has been given over weeks and months and has been shown to have multiple effects with improved exercise performance (hence use by elite athletes); improved cognitive performance in older people; vasodilation with reduced blood pressure; antiplatelet and anti-leucocyte effects and reduced platelet-leucocyte conjugation; improved endothelial function; reduced left ventricular volume; improved metabolic profile; and improved cardiac function. Beyond anti-inflammatory effects on blood cells, nitrite or nitrate reduce soluble pro-inflammatory factors including C-reactive protein, chemokine (C-X-C motif) ligand-1/2, endothelin-1, interleukins-1β/6/10/12p70, interferon-γ, monocyte chemoattractant protein and tissue necrosis factor-α. Dietary nitrate has profound metabolic effects and appears to have the potential for reversing the metabolic syndrome and have anti-diabetic effects through improving insulin sensitivity and lowering blood glucose levels. Overall, the pharmacological effects of beetroot juice have been demonstrated in younger and older people, and in people with cardiovascular disease, e.g. diabetes, obesity, hypertension, hypercholesterolaemia, heart failure and stroke. Importantly, inorganic nitrate (given as beetroot juice) may be taken by pregnant women. Experimentally, watermelon juice and chard gel may be used as an alternative source of dietary nitrate.

Phosphodiesterase-5 inhibitors
PDE5-inhibitors, such as dipyridamole and sildenafil, enhance the physiological effects of NO as mediated by cGMP. Whether these agents should have antimicrobial effects is unclear since they do not enhance NO levels per se; nevertheless, both drugs have exhibited antimicrobial activity (Table 7) and are being tested in COVID-19 trials (Table 9).

Stimulation of endogenous nitric oxide-dependent nitric oxide production
Endogenous NO production may also be stimulated externally. First, nasal breathing promotes the production of NO from the paranasal sinuses and this has bronchodilatory, vasodilatory and potential antimicrobial activities. This natural defence mechanism may be attenuated with mouth breathing, as occurs with increasing age and obesity. Second, ultraviolet radiation (UVA and UVB) stimulates the release of NO from both keratinocytes and melanocytes; NO has multiple effects including attenuation of free radical damage, melanogenesis, blood pressure lowering and potentially protection against skin infections.

Novel nitric oxide agents
Recent research has focussed on the development of new antimicrobial NO delivery systems and some examples are listed:

- NO microspheres, e.g. biodegradable poly (lactic-co-glycolic acid) spheres loaded with S-nitroso-N-acetyl-D- penicillamine.
- NO-releasing nanoparticles, with activity against Acinetobacter baumannii, C. albicans, Enterococcus faecalis, E. coli, K. pneumoniae, P. aeruginosa, S. aureus (MRSA), S. epidermidis, Trichophyton mentagrophytes.
- Modified chitosan, e.g. against Trypanosoma cruzi, E. coli, S. aureus, Streptococcus mutans.
- NO–metal complexes (zeolites), with activity against B. subtilis, Clostridium difficile, E. coli, P. aeruginosa, S. aureus (including MRSA).
- NONOates (diazeniumdiolates), e.g. with activity against C. albicans, E. coli.
NO coating of medical device surfaces and tubing,\textsuperscript{116,117} e.g. using S-nitroso-N-acetyl-D-penicillamine to kill \textit{Staphylococcus aureus} and \textit{P. aeruginosa}.

\textbullet{} RRx-001, a small molecule nitric oxide donor.\textsuperscript{118}

NO sources can also be categorised by whether administration is local (e.g. cutaneous nitrite or intranasal preparations), systemic (e.g. dietary nitrate, L-arginine or L-citrulline, oral isosorbide or sildenafil, sublingual GTN, intravenous GTN or SNP) or mixed local and systemic (transdermal GTN). Local administration allows high and potentially cidal concentrations of NO to be achieved without unwanted systemic effects. Intravenous formulations might allow for systemic infections to be treated.

\textbf{Relevance of in vitro studies to preclinical and clinical studies}

Most microbial studies presented above and in Tables 2-5 were performed \textit{in vitro} and involved either inducing the L-arginine/NO pathway with cytokines (e.g. interferon gamma [IFN-\(\gamma\)] and/or lipopolysaccharide [LPS]) or with NO sources (such as NO gas, nitrite, 3-morpholinosydnonimine, S-nitroso-L-acyetylpenicillamine or sodium nitroprusside). However, the inhibitory effect of NO on microbes \textit{in vitro} does not represent the complex biochemical environment that they face \textit{in vivo} including the presence of NO derivatives such as peroxynitrite, microbial production of NO, microbial resistance to NO and excess NO synthesis. Nevertheless, there are many \textit{ex vivo} and clinical examples where NO has been effective. These issues are now discussed.

\textbf{Nitric oxide for clinical infections}

\textbf{Oral health and gastrointestinal infections}

As already highlighted, oral bacteria (e.g. \textit{Corynebacterium pseudodiphtheriticum}, \textit{Fusobacterium nucleatum}, \textit{Nocardia spp.}, \textit{Prevotella melaninogenica}, \textit{S. aureus}, \textit{S. epidermidis}, \textit{Veillonella spp.}) are vital for the reduction of salivary nitrate to nitrite as part of the entero-salivary circulation; nitrite is further reduced to NO.\textsuperscript{119} This represents a symbiotic relationship between bacteria and the mammalian host; the host provides the nutrients and the environment in return for nitrite production,\textsuperscript{29} as in the absence of oxygen, nitrate and nitrite are commonly used by such bacteria as terminal electron acceptors for respiration.\textsuperscript{8}

Oral consumption of nitrate and the resulting increase in nitrite in the oro-pharynx leads to salivary alkalinisation (pH ~7.0 to 7.5)\textsuperscript{120} and so reduction in detrimental bacteria and caries.\textsuperscript{77} Similarly, nitrate supplementation was associated with increased oral \textit{Rothia} spp. and \textit{Neisseria} spp., and diminished oral \textit{Prevotella} spp. and \textit{Veillonella} spp.; in parallel, plasma nitrite levels rose and systemic blood pressure fell.\textsuperscript{121} Salivary nitrite production is related to the abundance of oral-nitrate-reducing bacteria.\textsuperscript{122} In contrast, bacteria and yeast, in particular \textit{Lactobacillus} spp., \textit{Streptococcus} spp. and \textit{C. albicans}, are key to the development of dental caries through the production of acid. Equally, antibiotics that kill nitrate-reductase-containing bacteria inhibit oral nitrite production and so increase the risk of oral thrush.\textsuperscript{123} Acidified nitrite has antibacterial activity against \textit{Helicobacter pylori in vitro},\textsuperscript{124} an experiment that likely mimics the scenario seen by these bacteria in the stomach after a nitrate/nitrite-rich meal.

\textbf{Cutaneous infections}

The skin is a potent source of nitric oxide and production is increased with exposure to sunlight (specifically ultraviolet radiation) sufficient to lower blood pressure.\textsuperscript{125,126} Hence, skin-derived NO may form a natural dermatological antimicrobial defence. Numerous studies have demonstrated that topical acidified sodium nitrite reduces cutaneous infections due to a variety of viruses and bacteria (Table 7) although prophylaxis had to be continued in some cases since NO suppressed replication without necessarily being viro-toxic.\textsuperscript{127–132} Inorganic nitrate has been used for the treatment of human papilloma virus.\textsuperscript{133} Phase II clinical trials have found that acidified nitrite in cream reduced \textit{Leishmania major/ tropica} amastigotes and promastigotes with a reduction in cutaneous leishmaniasis\textsuperscript{129} and increased cure rates in tinea pedis.\textsuperscript{127} Novel NO agents are in development to treat skin conditions (Table 8).

\textbf{Respiratory infections}

In animal and human experiments, NO substrate (L-arginine) and a NO donor (SNP) has been shown to improve the mucociliary activity of the upper respiratory tract\textsuperscript{134} suggesting a modulatory role for NO in nasal barrier function and clearance. Novel NO agents building on this observation are in development (Table 8).

Endogenous NO has potent pulmonary haemodynamic and bronchodilator effects physiologically. The importance of endogenous NO in preventing infection is apparent experimentally where inhibition of NO results in increased susceptibility to microbes including \textit{Leishmania} spp., \textit{Mycobacterium} spp. and \textit{Plasmodium} spp.\textsuperscript{135} Similarly, NO sources are used therapeutically, for example sildenafil in the management of pulmonary hypertension (Table 7).
In respect of airway epithelial cells, nitrite reduced *P. aeruginosa* biofilm growth.\textsuperscript{136} In infection, NO reduces pulmonary vascular resistance and intrapulmonary shunt, and improves oxygen partial pressure in patients with acute severe pneumonia.\textsuperscript{137} More specifically, inhaled NO improves arterial oxygenation enabling a reduction in inspired oxygen therapy and airway pressure support, and reduces lung infiltrates, in patients with severe acute respiratory syndrome (SARS).\textsuperscript{33} These findings continue after termination of NO therapy suggesting that NO has both pulmonary vasodilator and anti-SARS effects. Small uncontrolled clinical studies have suggested that iNO may be beneficial in COVID-19.\textsuperscript{138–142} iNO and novel NO agents are in development, primarily for COVID-19 at present (Tables 8, 9).

Dipyridamole, a phosphodiesterase 5 inhibitor, may also have similar beneficial effects in severe COVID-19.\textsuperscript{35} A phase II clinical trial found that L-arginine might have beneficial effects when given on top of conventional therapy for tuberculosis (Table 7).\textsuperscript{50}

Urinary tract infections

There may also be a role for dietary nitrate/inorganic nitrite in the prevention and treatment of urinary tract infections. Many of the lower urinary tract opportunistic organisms (*e.g.* *E. coli*) possess nitrate reductases, this forming the basis of urine dipstick detection of nitrite. In acidic urine conditions, nitrite is reduced to NO with toxicity to bacteria; for example, transferring nitrite-rich urine containing *E. coli* to a more acidic environment (*e.g.* pH 5.5) dose-dependently inhibited bacterial growth,\textsuperscript{143} an effect potentiated by vitamin C. The antibacterial potency is comparable to conventional antibiotics such as trimethoprim and nitrofurantoin.

Table 8. Examples of commercial development of novel nitric oxide donors/agents with efficacy against target disease and microbes (where relevant, last searched 15 March 2021).

| Target organism/disease                                      | Commercial company               |
|-------------------------------------------------------------|----------------------------------|
| NO gas for inhalation                                       | Beyond Air                       |
| NO gas for inhalation                                       | INOmax                           |
| NO-releasing solution for nebulisation (sodium nitrite and citric acid) | COVID-19                         |
| NO released from acidified nitrite via a semi-permeable membrane | Cutaneous *S. aureus, E. coli*\textsuperscript{257} |
| NO macromolecular scaffolds\textsuperscript{48,258}, e.g. NO-releasing cyclodextrins\textsuperscript{259} | *P. aeruginosa*                   |
| NO-releasing solution/gel (NORS2791)                        | Vast Therapeutics                |
| NO-releasing solution (NORS6491)                            | SaNOtize                         |
| NO-releasing nasal spray (NORS1002)                         | Novan                            |
| NO-releasing nasal lavage (NORS4002)                        | GeneOne Life Science             |
| NO-stimulating nasal spray (GLS-1200)                       | COVID-19                         |
| Nitric oxide generating lozenges (sodium nitrite)          | Nitric Oxide innovations         |
| NO-generating probiotic patches, *e.g.* based on Lactobacilli conversion of glucose to lactic acid, and acidification of sodium nitrite\textsuperscript{254,255} | *S. aureus*                      |
| Nitroreductase-activated release of NO, *e.g.* by O\textsuperscript{2-}(4-Nitrobenzyl) diazeniumdiolate\textsuperscript{261} or nitroaromatic-protected piperazine diazeniumdiolate\textsuperscript{262} | *E. coli*                        |
|                                                            | McGill University, Canada        |
|                                                            | Indian Institute of Science Education and research, Pune, India Colorado State University, Fort Collins, USA
| Trial name | NO source | Location | Design | Phase in population | Phase in hospital | Outcome | Sites | Rx days | Finish | Outcome Sites |
|------------|-----------|----------|--------|---------------------|-------------------|---------|------|---------|-------|----------------|
| COVID-19 prevention | Nitrate juice, oral | UK | Cluster | Phase II in care homes | New infection (any, including C-19) | New C-19 diagnosis | ADEPE | 30 | 06/21 | | |
| NO gas, inhaled | USA | CCS | DBPC | Phase II in healthcare staff | New C-19 diagnosis | Adverse events, PCR-positive infection | 2470 | 14 | 03/21 | | |
| GLS-1200 nasal spray (NO stimulant) | USA | DBPC | Phase II | Adverse events, PCR-positive infection | ?? | ? | 225 | | | | |
| COVID-19 treatment | L-arginine, oral | Italy | RCT | Phase II in hospital: on oxygen | Normalisation of P/F fraction | ?? | 230 | 10 | 12/21 | | |
| L-citrulline, iv | USA | DBPC | Phase II | Adverse events, PCR-positive infection | ?? | ? | 225 | | | | |
| Fiorentino et al. | (NCT04637906) | Italy | RCT | Phase II in hospital: on oxygen | Normalisation of P/F fraction | ?? | 230 | 10 | 12/21 | | |
| L-citrulline, iv | USA | DBPC | Phase II | Adverse events, PCR-positive infection | ?? | ? | 225 | | | | |
| Covino & others | (NCT04570384) | Italy | DBPC | Phase II in hospital: Moderate | Mechanical ventilation | ?? | 225 | 10 | 12/21 | | |
| NO gas, inhaled | China | Italy | DBPC | Phase II in hospital: severe C-19, PaO2/FiO2 <300 on air | PaO2/FiO2 >300 on air | ?? | 225 | 10 | 12/21 | | |
| Somberg et al. | (NCT04601077) | USA | RCT | Phase II in early COVID-19 | Hypotension, hospitalisation | ?? | 225 | 10 | 12/21 | | |
| NO nasal spray/irrigation | Canada | DBPC | Phase II in hospital: on oxygen | Duration of infectivity | ?? | ? | 225 | | | | |
| NO inhaled | China | Italy | DBPC | Phase II in hospital: on oxygen | Duration of infectivity | ?? | 225 | 10 | 12/21 | | |
| NO gas, inhaled | USA | DBPC | Phase II in hospital: severe C-19, PaO2/FiO2 <300 on air | Duration of infectivity | ?? | ? | 225 | | | | |
| NO inhaled | USA | DBPC | Phase II in hospital: on oxygen | Duration of infectivity | ?? | ? | 225 | | | | |
| Nebulised sodium nitrite and citric acid (RESP301) | UK | DBPC | Phase II/III hospitalised COVID-19 | Progression in WHO scale by ≥1 point | ?? | ? | 225 | | | | |
| Sildenafil | China | Open | Open | Phase II in hospital: pneumonia | Disease remission | ?? | 225 | 10 | 12/21 | | |

ARDS: adult respiratory distress syndrome; C-19: COVID-19; CCS: case-controlled study; DBPC: double-blind placebo-controlled; iv: intravenous; RCT: randomised controlled trial; SOFA: sequential organ failure assessment; TBC: to be confirmed.
This approach has been tested by filling urinary catheter retention balloons with nitrite and ascorbic acid, resulting in measurable amounts of NO outside the membrane and effectively killing two strains of E. coli in the surrounding urine. A similar approach found decreased bacterial counts and prevented biofilm formation by P. aeruginosa, K. pneumoniae, and Enterobacter cloace (but not E. coli or S. aureus).

Last, instillation of bacillus Calmette-Guerin (BCG, an attenuated strain of Mycobacterium bovis) into the bladder is used for the treatment of superficial/non-muscle invasive bladder cancer and carcinoma in situ. BCG induces long-term increases in NOS activity in urothelial cells and the formed NO is toxic to the malignant cells. The use of BCG to provide non-specific protection against SARS-CoV-2 is to be tested although vaccination in infancy does not appear to protect against COVID-19 in adults.

Other infections
Nitrate (usually KNO3) and nitrite (NaNO3) have been used for millennia to preserve food, especially meat and fish. Food preparation leads to reduction of nitrate to nitrite, and nitrite inhibits bacterial growth, especially Clostridium botulinum, a key and severe cause of neurotoxin poisoning. Additionally, nitrite adds colour to food and flavour (in part by overcoming rancid tastes) and is an antioxidant.

NO donors have also been investigated for eradicating or dispersing biofilm organisms. For example, GTN synergises with citrate and ethanol in eradicating biofilms (related to S. aureus, MRSE, P. aeruginosa and C. albicans) in an experimental catheter lock model. Similarly, isosorbide mononitrate synergised with antibiotics to disperse then kill S. aureus. An NO-releasing contact lens has been developed to treat microbial keratitis due to P. aeruginosa and S. aureus.

GTN may have improved outcome after infection in participants enrolled into the RIGHT-2 trial, a study where paramedics recruited patients with suspected stroke and randomised them to GTN versus sham. Overall, the trial was neutral. However, in a planned subgroup analysis of those participants with a final diagnosis of a non-stroke mimic, functional outcome was better with GTN. In a post hoc analysis of participants in this subgroup, GTN was associated with a better outcome in those with a final diagnosis of infections of the respiratory and urinary tracts which raises the possibility that NO was treating the infectious cause underlying the stroke mimic diagnosis.

The prostaglandin-cyclic adenosine-phosphodiesterase-3 system
As with the NO system, the prostaglandin-cyclic adenosine monophosphate-phosphodiesterase-3 (PG-CAMP-PDE3) system has similar vasculo-protective roles with anti-leucocyte, antiplatelet and anti-smooth muscle, and pro-endothelial effects. It is therefore interesting to note that prostaglandins (PGA1, PGJ2), including prostacyclin (PGI2 and analogues), have been reported to have antiviral effects. Whether drugs based on these or the PDE3 inhibitor, cilostazol, have efficacy against SARS-CoV-2 remains to be investigated. Further, endogenous NO and PGI2 work together in the vascular tree, and it is conceivable that their potential antimicrobial effects will similarly synergise. Their combination, in the forms of ISMN and cilostazol, have been tested after stroke but not yet reported for the prevention or treatment of infection.

Interaction between nitric oxide and vaccine efficacy
The interaction between diet, nutrition state and vaccine effectiveness has been assessed in multiple studies, principally in low–middle income countries where vaccination is paramount, especially in children, and yet where malnutrition may be widespread. In a systematic review and meta-analysis of observational studies and randomised controlled trials, there was little suggestion that malnutrition had any effect on vaccine responses; similarly, supplementation with vitamins and D, and iron and zinc, did not appear to modify responses. In preclinical studies, protein-energy malnutrition had limited influence on vaccine efficacy in mice. The effect of dietary nitrate levels on vaccine efficacy is unstudied.

If nitric oxide derivatives attenuate microbial infections, then the efficacy of vaccines based on live attenuated viruses and bacteria (such as measles, polio virus, BCG) might be attenuated by treatment with NO. Although there are many factors known to alter vaccine effectiveness (e.g. age), the effect of NO has not been studied.

Post-infection morbidity
Many infections cause long-term morbidity with chronic fatigue syndrome (CFS) and symptoms including fatigue, tiredness, myalgia, cognitive impairment and depression. Example associated microbes include Borrelia burgdorferi (Lyme disease), Chlamydia pneumoniae (community acquired pneumonia), Epstein–Barr virus (infectious mononucleosis), human herpes virus 6 (exanthema subitum), human immunodeficiency virus (AIDS), polio virus, SARS-CoV-1 virus (SARS), SARS-CoV-2 (long-COVID) and West Nile virus (fever). Although CFS may represent chronic or latent infection, it is more likely to reflect the presence of post-infectious chronic inflammation. Hypothetically, these
patients might benefit from inorganic nitrates in view of their positive effects on exercise performance (elite athletes take beetroot juice for this purpose) and cognition, \(^7^6,^9^5\) and potentially antimicrobial effects, a question that needs addressing (Table 7). A phase II trial of L-citrulline is studying this approach in patients with post-polio syndrome. \(^6^8\)

**Excess nitric oxide during infection**

During severe infection, sepsis (defined as “life-threatening organ dysfunction caused by a dysregulated host response to infection”) often develops. Septic shock is a subset of sepsis and is a leading cause of death worldwide. \(^1^6^9\) It manifests as hyper- or hypo-thermia, altered mental state, hypotension, tachycardia, tachypnoea, hypoxia, anuria and/or lactataemia. This can occur with many infections due to:

- **Gram negative bacteria**: Bacteroides fragilis, C. pneumoniae, Enterobacter spp., E. coli, Haemophilus influenzae, Klebsiella spp., Legionella spp., Neisseria meningitidis, Proteus spp., P. aeruginosa, Yersinia pestis. \(^6^3\)

- **Gram positive bacteria**: Clostridium spp., Enterococcus spp., Listeria monocytogenes, Staphylococcus spp., Streptococcus agalactiae/pneumoniae/pyogenes

- **Viral**: Adenovirus, Coronaviruses, Dengue viruses, Ebola virus, Enteroviruses, human immunodeficiency virus, Influenza virus (A and B), haemorrhagic fever viruses, Parechoviruses. \(^1^7^0,^1^7^1\)

- **Fungi**: Candida spp. \(^1^7^2\)

- **Protozoa**: Plasmodium falciparum, Schistosoma mansoni. \(^1^7^3\)

Typically, autoamplification of circulating cytokines (so-called cytokine storm) leads to excess NO synthesis, mostly derived from inducible NOS, leading to high circulating NO levels and the development of septic shock. In these circumstances, treatment with exogenous NO might be inappropriate. Trials of inhibiting endogenous NO synthesis with NOS-inhibitors in critically ill patients with sepsis have been reported although, disappointingly, did not improve outcome; indeed, the non-selective NOS-inhibitor, NG-methyl-L-arginine hydrochloride (L-NMMA, 546C88), was associated with increased death. \(^1^7^4\) It is not clear why inhibiting NO synthesis was ineffective but non-selective NOS inhibitors were used meaning that both toxic (iNOS) and beneficial (eNOS) sources of NO were inhibited; pharmacologically, such inhibitors will have reduced cardiac output, organ perfusion and tissue oxygenation. In the absence of licensed selective iNOS inhibitors, perhaps the analogous approach used in the management of hyperthyroidism using block (with carbimazole) and replace (thyroxine) might be effective, i.e. block NOS activity and replace with a low dose of a NO donor. That excess NO is dangerous does not mean that pharmacological doses of NO cannot be effective (Figure 1, Tables 6, 11) since all effective interventions in medicine have an inverted “U” dose response.

Some infections have opposing *in vitro* and *in vivo* responses to NO. For example activated macrophage-derived NO or NO donors such as SNAP reduced Trypanosoma brucei proliferation *in vitro* \(^1^7^5,^1^7^6\) whereas endogenous iNOS-derived NO suppressed protozoa-antigen specific T-cell proliferative responses and so worsened infection, at least in infected mice. \(^1^7^8\) Intracellular protozoal infections are unlikely to be affected in this manner since macrophage-derived NO would be able to act directly on pathogens such as Leishmania major. \(^1^7^7\)

Other infections do not appear, at least *in vitro*, to induce iNOS. For example, Cryptococcus neoformans failed to induce iNOS in primed macrophages, \(^1^7^7\) apparently due to a lack of TNF-α secretion, probably because the polysaccharide capsule masked the signal for TNF-α secretion. Interestingly, non-encapsulated mutants of C. neoformans did induce endogenous NOS.

High levels of iNOS activation were antimicrobial in studies of malaria. Based on monocyte-derived mRNA levels in circulating blood, uncomplicated malaria was associated with increased levels of iNOS activation in contrast to patients with severe malaria who had lower levels. \(^1^7^9\) The dual effects of NO in malaria, i.e. both low and high levels appear to be hazardous, are further reviewed. \(^1^8^0,^1^8^1\)

**Epidemics and pandemics**

Over recorded history, most epidemics and pandemics have resulted from viral infections including Ebola (viral haemorrhagic fever), influenza (H1N1, H2N2, H3N2, H3N8), HIV-1 (AIDS), polio (poliomyelitis), smallpox, yellow fever, zika or corona (OC43, MERS-CoV, SARS-CoV-1/2) viruses. Bacterial pandemics have resulted from *Vibrio cholerae* (cholera), S. enterica (typhoid fever) and *Yersinia pestis* (plague). Studies *in vitro* have reported findings suggesting that NO can reduce infection for some of these pathogens (Tables 2, 3) but information appears to be lacking for smallpox, yellow fever, zika and cholera (Table 10).
With multiple pandemics over the last 100 years, it is only inevitable that further ones will occur and some, like COVID-19, will comprise a "global catastrophic biological risk". Global pandemics will most likely be caused by a respiratory-spread virus that crosses over from animals such that humans have no inherent immunity to it. Likely candidates include orthomyxoviruses (especially influenza A viruses such as H7N9), paramyxoviruses (e.g. measles, mumps, croup), pneumovirus (e.g. human metapneumovirus), coronaviruses and picornaviruses (especially rhinoviruses and enteroviruses). All of these have had strains that have crossed from animals to humans. This emphasis on RNA viruses is because DNA viruses tend to have lower mutation rates and, therefore, evolve more slowly and are less likely to escape the human immune system within the first rounds of infection. Nevertheless, DNA viruses, such as pox or herpes viruses from great apes or monkeys, do have the potential to jump species. Non-viral causes of pandemics are less likely since most bacteria will be treatable with broad-spectrum antibacterial agents, most fungi are thermally restricted, and prions would require massive food contamination (and only spread slowly). Protozoa are usually thermally restricted although global warming may allow malaria to spread more widely in temperate zones.

Unfortunately, pandemics/epidemics may co-exist as seen with SARS-CoV-2 and dengue in Brazil, and both with S. enterica in Pakistan; in part, this reflects increasing travel with aircraft providing a portal for numerous microbes. Of theoretical concern was the potential for COVID-19 and epidemic influenza to co-exist during winter in the Northern hemisphere, this possibly leading to a dramatic increase in deaths. Nevertheless, 'flu rates were very low in both southern and northern hemisphere 2020 winters, presumably due to hands, face, space, mask and fresh air measures. All-in-all, the absence of a true broad-spectrum of antiviral agents is a major concern and a potential agent such as NO with antimicrobial effects that extend beyond viruses would be most welcome.

Implications for SARS-CoV-2 and COVID-19
One possible explanation for the observation that COVID-19 outcomes are worse in older people, males, black or Asian ethnicity, and those with co-morbidities such as diabetes, hypertension, stroke and chronic lung disease, is that these groups have lower vascular NO activity and so mount a sub-optimal host response against infection. Increasing NO availability is therefore a potential therapeutic strategy. Several NO sources have potential relevance to preventing and treating COVID-19. L-arginine, sodium nitrite, GTN, SNP, NO and dipyridamole each have clinical antimicrobial activity and can be administered, variously, orally, intravenously or as NO gas in the intensive care unit. Transdermal

![Figure 1. Schematic of concentration response curve for antimicrobial effects of nitric oxide.](image-url)
GTN, and oral ISMN, dipyridamole and sildenafil may be administered in the community or hospital. Of these, NO gas, dipyridamole and sildenafil are already being tested for preventing or treating COVID-19 (Table 9). It remains to be determined if increasing dietary nitrate may be a cost effective and safe intervention of widespread health relevance for the prevention of COVID-19 and, indeed, other emerging, pandemic, epidemic or endemic infections. Recent trial evidence provides indirect supporting evidence for the potential anti-SARS-CoV-2 effect of NO. First, dexamethasone and tocilizumab reduced death in patients in intensive care units,\textsuperscript{190,191} and these agents and NO share anti-inflammatory effects. And second, interferon-ß reduced the need for intensive care in COVID-19 patients;\textsuperscript{192} type I interferons increase iNOS activity and so have antimicrobial effects, as seen with \textit{L. major} and \textit{Burkholderia pseudomallei}.\textsuperscript{193,194}

Table 10. Future research questions relating to nitric oxide therapy for microbes.

| Question                                                                 | Microbe                                                                 | In vitro | In vivo | Clinical trials |
|--------------------------------------------------------------------------|------------------------------------------------------------------------|----------|---------|-----------------|
| What effect does NO have on so-far unstudied pandemic microbes?           | Viruses: smallpox, yellow fever, Zika. Bacteria: \textit{Vibrio cholerae} |          | +       |                 |
| What effect does NO have on so-far unstudied other microbes?             | Bacteria: Mycoplasma. Archaea.                                         |          | +       |                 |
| What effect does NO have on micro/macrotrombosis?                       | Viruses: Ebola. Influenza. MERS. SARS-CoV-1/2                           |          | +       |                 |
| What is the effect of timing on outcome in prevention or treatment of mild and severe disease? | Any                                                                     |          |         |                 |
| Could NO be used as a non-specific adjuvant to antimicrobial therapy (where septic shock is absent)? | Any                                                                     |          | +       | +               |
| Is the strategy of block (NOS inhibitor) and replace (NO donor) effective in septic shock? | Intensive Care Unit infections                                           |          | +       |                 |
| Do NO sources, e.g. dietary nitrate or NO donors, prevent/treat/improve outcome after COVID-19? | SARS-CoV-2                                                             |          | +       |                 |
| Do NO sources, e.g. dietary nitrate or NO donors, prevent and treat outcome after endemic \textit{flu}? | SARS-CoV-2                                                             |          | +       |                 |
| Do combined NO and PG sources have agonistic antimicrobial effects?      | Any                                                                     |          | +       | +               |
| Do NO sources reduce the efficacy of live attenuated vaccines?            | Measles virus, poliovirus, bacilli Calmette-Guerin                      |          | +       |                 |
| Do NO sources reduce chronic symptoms and improve quality of life after infection? | Lyme disease                                                           |          |         |                 |

NO: nitric oxide; PG: prostaglandin.

Table 11. Balance between potential beneficial and hazardous effects of NO sources in preventing and treating infections.

| Benefit                                  | Ineffective/Hazard                                                                 |
|------------------------------------------|-----------------------------------------------------------------------------------|
| \textit{In vitro}                        | Considerable static/cidal data                                                   |
|                                           | Limited neutral/negative data suggesting that there may be publication bias       |
| \textit{In vivo}                         | Some static/cidal data                                                            |
| Clinical                                 | Some positive phase II trials                                                    |
| Concentration                            | Moderate                                                                         |
|                                           | Low or very high (as in septic shock although NOS inhibitors ineffective)         |
|                                           | Microbial resistance to NO, e.g. synthesis of NO to resist oxidative stress        |
|                                           | Organic nitrates generate reactive NO species                                    |

GTN, and oral ISMN, dipyridamole and sildenafil may be administered in the community or hospital. Of these, NO gas, dipyridamole and sildenafil are already being tested for preventing or treating COVID-19 (Table 9). It remains to be determined if increasing dietary nitrate may be a cost effective and safe intervention of widespread health relevance for the prevention of COVID-19 and, indeed, other emerging, pandemic, epidemic or endemic infections. Recent trial evidence provides indirect supporting evidence for the potential anti-SARS-CoV-2 effect of NO. First, dexamethasone and tocilizumab reduced death in patients in intensive care units,\textsuperscript{190,191} and these agents and NO share anti-inflammatory effects. And second, interferon-ß reduced the need for intensive care in COVID-19 patients;\textsuperscript{192} type I interferons increase iNOS activity and so have antimicrobial effects, as seen with \textit{L. major} and \textit{Burkholderia pseudomallei}.\textsuperscript{193,194}
Discussion and conclusions

Nitric oxide is a fundamental molecule with wide-ranging and potent vascular, anti-platelet, anti-inflammatory and tumoricidal effects. Further, there is a large volume of literature spanning the last 30+ years demonstrating that NO also has potent in vitro antimicrobial effects on a wide variety of viruses, bacteria, protozoa, fungi and yeasts; these are supported by a modest number of in vivo studies. Further, several positive clinical phase II trials of NO have been reported in viral, bacterial, protozoa and fungal infections, these relating particularly to skin and respiratory infections administered by cream and gas respectively. Although not from randomised trials, there is also evidence that dietary nitrate modifies the oral biome and so reduces dental caries.

However, these results cannot be considered persuasive on their own. First, few neutral or negative studies have been reported suggesting that there may be a risk of publication bias. Second, conflicting data in some dual-protocol studies with positive in vitro and neutral/negative in vivo data suggest that although NO is antimicrobial per se, the local tissue environment may overcome or reverse this effect. Third, organic nitrates can suffer from tolerance and may lead to the generation of reactive NO species such as peroxynitrite and S-nitrosothiols which might exacerbate rather than attenuate infection. Fourth, resistance may develop although this seems unlikely to be a generic issue, not least because NO levels can change, and be changed, much faster than any microbe can raise defensive mechanisms. Fifth, some microbes can produce their own NO and use this to resist the oxidative stress induced by external NO and its derivatives. Sixth, excess NO production is associated with the development of septic shock which might suggest that any NO is ineffective. Potentially, unsuccessful trials of non-selective NOS-inhibitors in severe sepsis may have confused the issue, perhaps by suggesting that treatment with NO is not important in infection. Seventh, positive clinical studies have been performed in environments where very high local concentrations of NO can be achieved and without the risk of reactive responses, in particular on the surface of tissues such as cream on the skin, dietary nitrate in the mouth, nitrite in the stomach, NO gas in the lungs and nitrite in the bladder; whether NO is effective as an antimicrobial within tissues and the vascular tree remains to be determined.

There are many sources of NO suitable for studying the prevention and treatment of milder infections in the community and hospital (e.g. topical sodium nitrite, oral NO donors such as ISMN, or oral PDE5 inhibitors such as dipyridamole or sildenafil), and treatment of serious infections in hospital (e.g. intravenous L-arginine, sodium nitrite or NO donors such as GTN or SNP, of NO gas). NO may also be delivered via a high nitrate diet, thus offering a widely available and inexpensive public health approach to potentially reducing and attenuating the severity of infections worldwide. This approach has the added advantage that such diets are already known to reduce vascular disease and some cancers, and possibly other inflammatory diseases and dementia.

In summary, the wealth of in vitro data suggest that NO has generic antimicrobial effects. However, some data suggest that NO may be ineffective or even hazardous and these reinforce our view for the need for large scale clinical trials of NO donors in the community and hospitals to prevent and treat infections. Although such studies need to focus urgently on the COVID-19 pandemic (especially with the lack of broad spectrum antiviral agents188), other pathogens also need to be targeted. However, patients with established septic shock should not be administered NO donors to avoid exacerbating vascular collapse. One utopian vision would be demonstration that high dietary nitrate intake produces pre- or post-exposure prophylaxis against infections and their severity in the community whilst NO donors are effective antimicrobial treatments for use by general practitioners and in hospitals.

Data availability
No data are associated with this article.

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Nitric oxide (NO) has been used in many different clinical settings over the years. The approval of inhaled NO for the treatment of pulmonary hypertension of the newborn has been a game changer and has since then saved many lives. NO has failed to prove a significant outcome improvement in patients suffering from Acute Respiratory Distress Syndrome (ARDS).

In this review, the authors have focused on the not-widely known broad antimicrobial role of NO. Although the antimicrobial effects are known for more than two decades, there has not been a systematic review of the topic. In addition to the well-written and compelling text, I have some suggestions, which I believe will add to the review.

Inhalation of NO is a main source of exogenous NO and has been used for almost three decades. For a long time, the administration of inhaled NO has been limited to mechanically ventilated patients in the ICU. However, in recent years the development of devices enabling pulsed generation of NO out of ambient air and devices developed during the surge of SARS-CoV2 allow the use in awake patients. A recently published review summarizes the different delivery methods of inhaled nitric oxide in intubated and non-intubated patients and should be added to this review 1.

Another important aspect worth mentioning in a separate paragraph is the use of inhaled nitric oxide in patients suffering from cystic fibrosis. These are a special patient group for studying new therapeutic approaches. The lungs of these patients are often colonized by multiresistant hard-to-treat gram negative bacteria such as burkholderia and pseudomonas species.2,3,4

In this context, it is worth mentioning that there seems to be some evidence that inhaled NO, in addition to its beneficial effect on mucociliary clearance, dispersal of biofilm, and bronchiolysis,
improves antibiotic susceptibility in resistant bacteria.\textsuperscript{5,6}

One limitation of high dose NO treatment is the production of methemoglobin. However, there are different strategies to minimize methemoglobin levels. Intermittent delivery of inhaled NO helped to minimize metHb concentrations. In addition, in-vitro data suggest that intermittent delivery of high dose NO increases the overall antibacterial effect.\textsuperscript{7}

Another approach is the co-administration of high dose NO and methylene blue, which reduces methemoglobin to hemoglobin and helps limit methemoglobin production, and allowed longer high dose NO administration.\textsuperscript{8}

A very compelling clinical use might be the administration of inhaled NO as a prevention therapy for infections of the lung in explanted lungs in the context of a lung transplant. Ex-vivo perfusion and co-administration of high dose inhaled NO, similar to a high dose of Colistin e.g. during transportation might be a novel strategy to reduce complications after transplantation.\textsuperscript{9}

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\textbf{Is the topic of the review discussed comprehensively in the context of the current literature?}
Yes
Are all factual statements correct and adequately supported by citations?
Yes

Is the review written in accessible language?
Yes

Are the conclusions drawn appropriate in the context of the current research literature?
Yes

**Competing Interests:** No competing interests were disclosed.

**Reviewer Expertise:** ARDS, Immunology

I confirm that I have read this submission and believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard, however I have significant reservations, as outlined above.

Reviewer Report 30 May 2022

https://doi.org/10.5256/f1000research.78470.r138631

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Jan Mohammad Mir
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The manuscript under review represents a study of potential interest. Though several review articles of similar themes have already been published. This article however introduces NO as a one-man army for broad-spectrum bioactivity. Therefore, I suggest the following modifications to make the article more interesting to the readership:

1. Introducing lines in the introduction must be about human physiological effects of NO, and later defense by microbes and defense against microbes using NO be highlighted.

2. NO is a pleiotropic signaling molecule: given in the introduction should be made more comprehensive, and distinctive by using "Gasotransmitter" concept as well (reference 1).

3. 2nd paragraph “As people age, endothelial-derived vascular NO levels fall and so vascular function declines causing relative endothelial dysfunction, pro-platelet, and pro-inflammatory effects, and increased smooth muscle proliferation." needs literature support.

4. NO is a small diatomic hydrophobic colourless gas that diffuses easily and has a short half-life. Specify this line...

5. "In reality, the chemistry of NO is more complex and it exists in several redox forms:
nitrosonium cation (NO\(^+\)), NO (NO\(^*\)), and nitroxyl anion NO\(^{--}\)……

Discuss metabolic or biological fate only.

6. This part is suitable for introduction “As an inorganic molecule, its central role in biology as a signalling molecule was only discovered in the 1980s,\(^{24}\) the identification of which led to the Nobel Prize for Physiology and Medicine in 1998.\(^{25}\) Ironically, the medicinal use of NO in the form of glyceryl trinitrate (GTN) for angina prophylaxis antedates the modern understanding of the biological synthesis and role of NO by more than a century.\(^{26}\)"

7. The chemistry and biology section needs updating.

8. The most important/major modifications: give the colourful schemes showing the mechanism of action of NO against the target diseases. Also, the introduction, main discussion, and conclusion must be redrawn to highlight "NO as a treatment option", the clinical trials discussion, the use of several NORMS (NORM-1, NORM-2, etc), the effect of NO-carriage system, Natural NO-sources, synthetic NO-sources with limitations, Inorganic versus organic forms, etc (eg., in the review publications as given\(^2,^3\).

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Is the topic of the review discussed comprehensively in the context of the current literature?
No

Are all factual statements correct and adequately supported by citations?
Partly

Is the review written in accessible language?
Yes

Are the conclusions drawn appropriate in the context of the current research literature?
Partly

**Competing Interests**: No competing interests were disclosed.

**Reviewer Expertise**: Coordination, Bioinorganic and Computational Chemistry.

I confirm that I have read this submission and believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard, however I have
significant reservations, as outlined above.

Reviewer Report 25 October 2021
https://doi.org/10.5256/f1000research.78470.r97610

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The authors have adequately addressed my original concerns. The revised submission is acceptable.

Is the topic of the review discussed comprehensively in the context of the current literature?
Partly

Are all factual statements correct and adequately supported by citations?
Partly

Is the review written in accessible language?
Partly

Are the conclusions drawn appropriate in the context of the current research literature?
Partly

Competing Interests: No competing interests were disclosed.

Reviewer Expertise: Pharmacologist with a strong background in biochemistry and intermediary metabolism.

I confirm that I have read this submission and believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard.

Version 1
Reviewer Report 27 September 2021
https://doi.org/10.5256/f1000research.54420.r93464
Nitric oxide (NO) is one of the ten smallest molecules found in nature. It is released intracellularly when L-arginine is oxidized by the enzyme nitric oxide synthase (NOS), of which there are three isoforms. Neuronal (nNOS, NOS1) and endothelial (eNOS, NOS3) are constitutive calcium-dependent forms of the enzyme regulated by negative feedback and release low fluxes of NO over a short period regulating neural and vascular function, respectively. The third isoform (iNOS, NOS2) is calcium-independent, inducible, produces supra-physiological concentrations of NO, and is involved in immune surveillance. NO can also be produced through the reduction of nitrite/nitrate under low oxygen conditions.

NO has important roles in normal physiology, exemplified by regulation of vascular relaxation, control of inflammation by inhibiting NF-κB activation, and suppression of pro-inflammatory mediators in mast cells, macrophages, and vascular smooth muscles. In addition, NO regulates blood flow, modulates platelet and leukocyte activation, adhesion, and aggregation.

In this review, the authors have focused on NO's role in defense against multiple microbial pathogens [including viruses, bacteria, SARS-CoV (COVID-19), protozoa, and fungi/yeast]. In addition, they have highlighted the possibility that exogenous NO might have therapeutic potential as a broad-spectrum antimicrobial. Finally, they have also summarized various NO donating/releasing platforms.

In general, I do not think that there is a "right" or "wrong" way of presenting a review, as long as it is accurate, balanced, and gives adequate historical background. I found this review to be quite compelling and of interest to those starting in this field and an update for the seasoned investigators. In short, it is an authoritative review. For the novice, the review gives the directions for further in-depth reading. Finally, I would like to make some suggestions to the text for the authors to consider as I believe it would add to the overall body of this work.

- Throughout the text, when you talk about “NO may be taken as dietary substrate (inorganic nitrate, L-arginine)....", please also discuss L-citrulline as a source of NO. We recently reviewed this topic and you may want to use this review as a source to expand on this.

- In Table 1, please add L-citrulline as a substrate and give the appropriate biochemical equation.

- Under Therapeutic inorganic nitrite and nitrate, please consider adding the use of nitrite/nitrate in animal models of wound healing. Refer to Afzali et al. 2020 for an introduction to this.

- Under novel nitric oxide agents, please add RRx-001, which is a novel NO modulator.
As summarized in the review, dietary intake of foods such as beetroot that are rich as a source of nitrate have many health benefits. And as mentioned, "Dietary nitrate has profound metabolic effects and appears to have the potential for reversing the metabolic syndrome and have anti-diabetic effects.". However, although the beneficial metabolic effects of inorganic nitrate and nitrite in type 2 diabetes mellitus have been documented in animal experiments, this is not the case for humans. Perhaps this should be mentioned on page 18 of the review.

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1. Bahadoran Z, Mirmiran P, Kashfi K, Ghasemi A: Endogenous flux of nitric oxide: Citrulline is preferred to Arginine. Acta Physiol (Oxf). 231 (3): e13572 PubMed Abstract | Publisher Full Text
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Is the topic of the review discussed comprehensively in the context of the current literature?
Yes

Are all factual statements correct and adequately supported by citations?
Yes

Is the review written in accessible language?
Yes

Are the conclusions drawn appropriate in the context of the current research literature?
Yes

Competing Interests: No competing interests were disclosed.

Reviewer Expertise: Pharmacologist with a strong background in biochemistry and intermediary metabolism.

I confirm that I have read this submission and believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard, however I have significant reservations, as outlined above.

Author Response 14 Oct 2021
**Philip Bath**, University of Nottingham, Nottingham, UK

**Reviewer 1 responses**

We thank Dr. Kashfi for his most helpful and considered comments and respond to each as below.

In this review, the authors have focused on NO's role in defense against multiple microbial pathogens [including viruses, bacteria, SARS-CoV (COVID-19), protozoa, and fungi/yeast]. In addition, they have highlighted the possibility that exogenous NO might have therapeutic potential as a broad-spectrum antimicrobial. Finally, they have also summarized various NO donating/releasing platforms.

In general, I do not think that there is a "right" or "wrong" way of presenting a review, as long as it is accurate, balanced, and gives adequate historical background. I found this review to be quite compelling and of interest to those starting in this field and an update for the seasoned investigators. In short, it is an authoritative review. For the novice, the review gives the directions for further in-depth reading. Finally, I would like to make some suggestions to the text for the authors to consider as I believe it would add to the overall body of this work.

- Throughout the text, when you talk about “NO may be taken as dietary substrate (inorganic nitrate, L-arginine)...”, please also discuss L-citrulline as a source of NO. We recently reviewed this topic, and you may want to use this review as a source to expand on this.

  **We have added L-citrulline wherever dietary L-arginine is mentioned (abstract, text and tables), and added two references: Flam et al. 2007, Bahadoran et al. 2021.**

- In Table 1, please add L-citrulline as a substrate and give the appropriate biochemical equation.

  **We have added L-citrulline as a substrate and the relevant equations.**

- Under Therapeutic inorganic nitrite and nitrate, please consider adding the use of nitrite/nitrate in animal models of wound healing. Refer to Afzali et al. 2020 for an introduction to this.

  **We have added a comment relating to the reference of Afzali et al. 2020, and the reference itself.**

- Under novel nitric oxide agents, please add RRx-001, which is a novel NO modulator.

  **We have added a comment relating to RRx-001 using a reference from Oronsky et al. 2020, and the reference itself.**

- As summarized in the review, dietary intake of foods such as beetroot that are rich as a source of nitrate have many health benefits. And as mentioned, "Dietary nitrate has profound metabolic effects and appears to have the potential for reversing the metabolic syndrome and have anti-diabetic effects.". However, although the beneficial metabolic effects of inorganic nitrate and nitrite in type 2 diabetes mellitus have been documented in animal experiments, this is not the case for humans. Perhaps this should be mentioned on page 18 of the review.

  **We have added two references relating to beetroot juice improving insulin sensitivity and reducing blood glucose (Wootton et al. 2014, Beals et al. 2017).**
**Comments on this article**

**Version 1**

**Author Response 19 Oct 2021**

**Philip Bath**, University of Nottingham, Nottingham, UK

We thank Dr Taylor-Robinson for his comment reminding us of the importance of malaria and long history of research into the importance of nitric oxide with it having both protective and anti-immunological effects; we have added two references for this (Taylor-Robinson & Smith 1999, Shikani et al. 2012).

**Competing Interests:** Response from Philip Bath, lead/coordinating author.

**Reader Comment 12 Jul 2021**

**Andrew Taylor-Robinson**, Central Queensland University, Brisbane, Australia

While this is an otherwise authoritative review, an important oversight is the only one brief mention afforded to the role of nitric oxide (NO) and its various derivatives in host immunity to the malaria parasite, *Plasmodium*, a citation from as recent as 2013 (ref. 228).

Much is known of the protective - and pathological - effects triggered by NO metabolites in the mammalian immune response to asexual plasmodia, both during uncomplicated infection of the peripheral blood (PB) and cerebral malaria (CM) sequelae of parasitised erythrocyte sequestration.

Investigation of NO during malaria infection extends back to the late 1980s and throughout the 1990s, principally in murine models including *P. berghei* (mostly for CM) and *P. chabaudi* (mostly for PB). In many aspects, the pioneering research of the groups of Langhorne, Phillips, Stephenson, Clark and Hunt, among others, led the field for what was then known about the role of NO in immunity to protozoan infection, as well as more broadly.

The implications of this collective body of work have been extrapolated extensively to other intracellular pathogens as they were reached by exploiting a tractable system for dissecting the evidently subtle balance between immune protection and immunopathology. The main findings remain very relevant to a contemporary consideration of the part played by NO in prevention and treatment of infectious diseases.

**Competing Interests:** I declare no competing interests.
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