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# Small-Molecule NOX Inhibitors: ROS-Generating NADPH Oxidases as Therapeutic Targets

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**Abstract**

NOX NADPH oxidases are electron-transporting membrane enzymes whose primary function is the generation of reactive oxygen species (ROS). ROS produced by NOX enzymes show a variety of biologic functions, such as microbial killing, blood pressure regulation, and otoconia formation. Strong evidence suggests that NOX enzymes are major contributors to oxidative damage in pathologic conditions. Blocking the undesirable actions of NOX enzymes, therefore, is a therapeutic strategy for treating oxidative stress-related pathologies, such as ischemia/reperfusion tissue injury, and neurodegenerative and metabolic diseases. Most currently available NOX inhibitors have low selectivity, potency, and bioavailability, precluding a pharmacologic demonstration of NOX as therapeutic targets *in vivo*. This review has two main purposes. First, we describe a systematic approach that we believe should be followed in the search for truly selective NOX inhibitors. Second, we present a critical review of small-molecule NOX inhibitors described over the last two decades, including recently published patents from the pharmaceutical industry. Structures, activities, and *in vitro/in vivo* specificity of these NOX inhibitors are discussed. We conclude that NOX inhibition is a pertinent and promising novel pharmacologic concept, but that major efforts will be necessary to develop specific NOX inhibitors suited for clinical application. *Antioxid. Redox Signal.* 11, 2535–2552.

## Introduction: NOX Enzymes and Their Distribution/Regulation

THE NOX NADPH oxidases comprise a family of reactive oxygen species (ROS)-producing enzymes that is increasingly recognized as a source of oxidative stress in many disease settings. Whereas NOX2 (also known as gp91$^{phox}$), the phagocyte oxidase, has been known for several decades as the enzyme responsible for the oxidative burst and associated microbicidal activity, the other members of the gene family have been identified only recently. The NOX family now consists of seven members (NOX1, NOX2, NOX3, NOX4, NOX5, DUOX1, and DUOX2), each with a distinct tissue distribution. Since the discovery that NOX enzymes are not limited to white blood cells, an exponential increase in scientific reports describe how NOX enzymes are responsible for increased ROS generation in numerous pathologic conditions, such as hypertension, ischemia/reperfusion, diabetes, cardiovascular diseases, and neurodegeneration (59). The elevated ROS production has been linked to the pathobiology of many of these conditions (59). The core catalytic domains of all seven NOX isoforms share similar structures, and their only known biochemical function is the generation of ROS. The basic catalytic subunit of NOX contains a C-terminal dehydrogenase domain featuring a binding site for NADPH and a bound flavin adenine nucleotide (FAD), as well as an N-terminal domain consisting of six transmembrane alpha helices that bind two heme groups. On activation, cytosolic NADPH transfers its electrons to the FAD, which in turn passes electrons sequentially to the two hemes and ultimately to molecular oxygen on the opposing side of the membrane, to form the superoxide anion ($O_2^-$) (22).

Although all seven NOX isoforms catalyze the reduction of molecular oxygen, they differ in their tissue distribution, their subunit requirements, domain structure, and the mechanism by which they are activated. In the case of NOX2, the activation mechanism is well described: on activation, the regulatory subunit p47$^{phox}$ is phosphorylated and translocates to the membrane, in the form of a complex that also contains p67$^{phox}$ and p40$^{phox}$. Once at the membrane, the cytosolic complex binds to the transmembrane cytochrome unit comprising both NOX2 and the closely associated p22$^{phox}$. Independently, the GTP-binding protein Rac also moves to the membrane, and the combination of regulatory subunits

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induces activation. Like NOX2, NOX1 and NOX3 require $p22^{phox}$, as well as association with cytosolic regulatory components ($p47^{phox}$/$p67^{phox}$ or their homologues NOXO1, NOXA1) $p40^{phox}$ and Rac. NOX4 requires $p22^{phox}$, but not the cytosolic regulatory factors (10). NOX5 and the DUOXes are activated by elevation of intracellular $Ca^{2+}$, which binds directly to N-terminal EF-hand domains.

## NOX Inhibitors: Historical Overview

During the past decade since the discovery of new homologues of NADPH oxidases, interest has greatly increased in identifying novel and specific NOX inhibitors, both as powerful tools to gain information about enzyme function and as potential therapeutic agents. The majority of NOX inhibitors were first identified and characterized based on their ability to block the neutrophil oxidative burst mediated by NOX2 (also commonly referred to as the respiratory burst oxidase or the phagocyte oxidase). Although these inhibitors lack specificity, they have provided important information about the NOX family as a whole. For example, flavoprotein inhibitors (diphenyleneiodonium, quinacrine) and analogues (5-Deaza-FAD), heme ligands (bipyridyl and benzylimidazole) (86), and NADPH analogues (ADP, cibacron blue) were used to determine the now widely accepted order of the electron transfer through the NOX2 prosthetic groups (31). Holland *et al*. (41) used multiple compounds known to inhibit the phagocyte oxidase to demonstrate the presence of a superoxide-producing activity in vascular endothelial cells (now known to be due not only to NOX2, but also to NOX4).

The utility of these early NOX inhibitors is limited by a number of factors. NOX2 is expressed in many tissues, so a wide range of effects might be expected from a general inhibition of NOX2. Also, many of these inhibitors are not isoform specific, so several or all NOX isoforms would be expected to be blocked. Finally, the inhibitors are often not specific for NOX, either because they block upstream pathways, or because they act directly on both NOX and other targets.

The classic inhibitor diphenyleneiodonium (DPI), has been useful not only to elucidate the oxidative burst of phagocytes (30), but also as an inhibitor of all NOX isoforms. However, because its chemical mechanism of inhibition involves accepting an electron from flavin, followed by covalently reacting with the enzyme or its prosthetic groups, DPI interferes not only with NOX enzymes, but also with many other flavin-dependent enzymes, such as nitric oxide synthase (NOS) and NADH coenzyme Q reductase.

Specificity of action is a major challenge in the NOX field in general. In 1990, Cross (20) assembled a then-comprehensive list of NOX2 inhibitors, identifying >120 such compounds. In a more recent article, the same author stated that >350 inhibitors have been described (22). However, a large number of the compounds listed lack specificity, as they do not directly block the enzyme, but rather interfere with upstream signal-transduction pathways (these include protein kinase inhibitors for NOX2 and NOX5 or molecules that interfere with the renin-angiotensin system for NOX1) or act as antioxidants or ROS scavengers (for example, superoxide dismutase and peroxidase mimetics, *N*-acetyl cysteine, dimethyl sulfoxide). Other compounds exert a direct inhibitory effect on the oxidase complex, but also block other enzyme systems sharing common structural binding sites and therefore affect other enzymatic activities (cytochromes p450, mitochondrial electron-transport chain, and NOS, among others). Thus, while we await the development of new isoform-specific NOX inhibitors, the existing NOX inhibitors can be useful when combined with careful controls and informed interpretation.

This review describes NOX inhibitors that were discovered in the last two decades, with the exception of DPI. The focus here is limited to small-molecule inhibitors for which a sufficient body of evidence is documented for a real inhibitory action on the enzyme (as described in detail subsequently) and a high affinity toward the target ($IC_{50} < 100\ \mu M$). Therefore, we have not included peptide inhibitors such as gp91tat, or inhibitors with low potency such as AEBSF (4-(2-aminoethyl)-benzenesulphonyl fluoride), with its $IC_{50}$ of $\sim 1$ m$M$ (26).

Cell-permeable NOX peptide inhibitors (*e.g*., gp91-dstat and PR-39 and some Rac peptide inhibitors) have been used extensively, and these were recently reviewed elsewhere (92). Notably, a number of limitations are associated with the use of such cell-permeable peptides as therapeutic agents, and additional studies are needed to characterize fully their modes of action as probes of NOX function.

## NOX Inhibitors *Versus* Oxidative-Burst Inhibitors

Identification of molecules that block NOX and have potential *in vivo* use is a major challenge. However, the potential benefits for therapeutic use as well as for basic research are enormous. NOX activity is often measured indirectly by using probes whose colorimetric, fluorescent, or chemiluminescent chemical properties change when they interact with ROS, mainly superoxide or hydrogen peroxide, which forms rapidly *via* superoxide dismutation, both spontaneously and through the catalytic action of superoxide dismutases (89). This type of approach is technically straightforward, and it can be used with high-throughput screening to identify small molecules with NOX inhibitory effect. However, a number of considerations apply.

### *Inhibition of an alternative source of ROS*

NOX is not necessarily the only source of ROS, particularly in complex systems that may also involve xanthine oxidase, cytochrome P450, the mitochondrial electron-transport chain, and uncoupled NOS, which is a substantial source of ROS production, especially in disease states (87). Therefore, care must be taken when attributing a reduction in the amount of ROS detected to inhibition of a NOX rather than some other source of ROS.

### *Detection of ROS versus true enzyme activity*

Many of the probes selectively detect a particular chemical species of ROS, and a decrease in the signal may not reflect a true decrease in the activity of the enzyme. Rather, the decreased signal may reflect a decrease in the availability of that species, through either ROS scavenging or metabolism.

### *Inhibition through indirect pathways*

Detectable NOX activity may be decreased independently of direct effects on the enzyme itself through indirect or nonspecific pathways, such as interfering with the cell-signaling pathways leading to activation, or even physical disruption of

the membrane with loss of cell viability. Inhibitors that act upstream to block NOX activation may nonetheless have good therapeutic potential, but they cannot be considered true NOX inhibitors.

*Nonspecificity or interference with the assay*

Many of the assays currently in use are based on detecting free radical species. As such, they are inherently susceptible to artifacts from other sources of free radicals that are present in the system. In addition, the ROS generated may not be detected because of an interfering reaction between the inhibitor and the detection probe. Hence, ideally two or more different methods should be used to confirm the effectiveness and specificity of candidate inhibitors.

## What Are the Criteria for Selecting a NOX Inhibitor *In Vitro*?

Multiple lines of evidence for NOX inhibition are required to exclude a pleiotropic effect on NOX activity. In that respect, the work from the group of Wang (China Medical College, Taiwan) (43, 44, 110, 112, 115) is notable as an example of the approaches that can be used to investigate the action of natural compounds on the neutrophil oxidative burst (*i.e.*, NOX2 activity) and to discriminate between compounds that act directly on the enzyme *versus* the activation process upstream of NOX2. It is not sufficient to use a single assay to evaluate NOX inhibition, but rather a sequence of assays, as summarized in Fig. 1.

*Inhibitor evaluation: detecting the inhibition of NOX-mediated superoxide generation in intact cells*

Two main possible approaches are available: either detecting the reaction product (*i.e.*, ROS or a downstream by-product) or measuring substrate consumption (*i.e.*, oxygen or NADPH).

Measurement of the amount of ROS produced. This can be done by techniques that detect the ROS species directly, by using colorimetric, fluorescent, or luminescent probes (*e.g.*, superoxide dismutase-inhibitable reduction of ferricytochrome *c*, horseradish peroxidase–catalyzed conversion of Amplex red to resorufin, or luminol oxidation, respectively), the use of spin-trap probes for detecting free-radical species by electron spin resonance [*e.g.*, ACP (1-acetoxy-3-carboxy-2,2,5,5-tetramethylpyrrolidine); for review, see ref. 27], or by electrodes that detect hydrogen peroxide (57). Alternatively, ROS can be measured indirectly by methods that detect their reaction products, such as lipid peroxidation by the detection of thiobarbituric acid–reactive substances (TBARS) (118) or DNA damage by 8-hydroxydeoxyguanosine (14). The advantages and disadvantages of the techniques used for the detection of ROS generation were recently reviewed (27, 89).

Neutrophils are a very convenient system with which to study NOX2, as it is the major NOX isoform expressed, it produces large quantities of ROS on activation, and little or no ROS are produced from other sources. In addition, NOX2-deficient neutrophils (from patients with the inherited

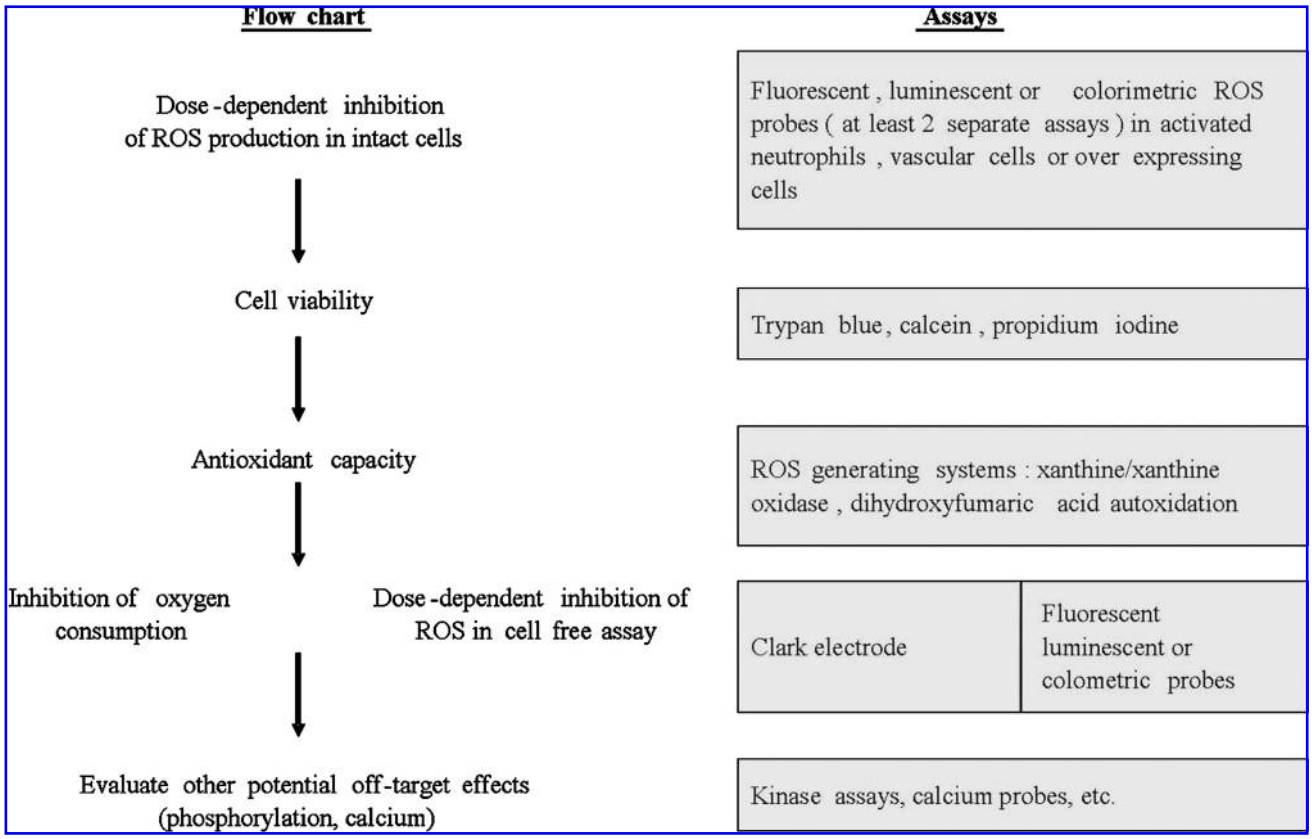


**FIG. 1. Flow chart representing the sequence of experiments and the most common techniques required to demonstrate NOX inhibitory activity *in vitro*.**

NOX2-deficiency disorder chronic granulomatous disease or from gene-knockout mice) can be used as controls. The study of other NOX isoforms is more challenging. Primary cells or continuous cell lines that endogenously express a single NOX isoform apart from NOX2 are not easily obtained or validated. In addition, the endogenous expression levels of these isoforms are often low, and the rates at which ROS are produced by these isoforms may also be different. For these reasons, transfected cells overexpressing specific NOX isoenzymes are an attractive well-defined alternative to the use of cells that endogenously express NADPH oxidases. A variety of transfection systems developed to characterize the activity and regulation of various NOX isoforms can serve to test NOX inhibitors (40, 94, 95).

Measurement of substrate consumption. Oxygen consumption can be done by using a Clark-type oxygen electrode. Because this method measures the disappearance of the electron acceptor, it can be used to rule out an effect that results from oxidant scavenging. However, neither of these methods can definitively attribute the decrease in ROS to an inhibition of NOX enzymes, unless the source of ROS generation in the cells used is already known and validated. A limitation of this method is that it is several orders of magnitude less sensitive than fluorescent and luminescent probes that detect ROS. Another method involves the measure of the rate of NADPH oxidation during the oxidative burst (13), which likewise has a lack of sensitivity and specificity.

#### *Inhibitor evaluation: detecting the scavenging capacity and toxicity*

A decrease in detection of ROS or their by-products could occur as a result of ROS scavenging effects of the compound rather than NOX inhibition. Cytotoxic effects would affect both ROS detection and the substrate consumption signal obtained with the Clark electrode.

Superoxide scavenging activity of the compound. Some compounds may artefactually appear to inhibit NOX because they scavenge ROS before they can be detected, thereby competing with the detection probe. In addition to the oxygen-electrode approach mentioned earlier, antioxidant or scavenging effects may be demonstrated by measuring nitroblue tetrazolium (NBT) reduction during dihydroxyfumaric acid autooxidation (35) or by using the xanthine/xanthine oxidase superoxide-generating system (112, 113). When interpreting results, one must consider that an antioxidant effect *per se* does not in and of itself indicate that the compound does not also inhibit NOX (*e.g.*, apocynin). In addition, for the xanthine/xanthine oxidase assay, compounds that directly inhibit xanthine oxidase could also inhibit NOX (*e.g.*, DPI).

Toxicity. Cell-viability assays, such as trypan blue, calcein, or others, are important to ensure that apparent inhibition is not due to a cytotoxic effect of the compound.

#### *Inhibitor evaluation: detecting direct interaction of the compound with NOX catalytic subunits*

Measurement of superoxide generation in a broken-cell assay. In whole cells and tissues, it is not always clear whether NOX enzymes are the source of the ROS that are being inhibited, and even in well characterized cells in which this may be known, it is often not possible to determine whether a putative inhibitor is acting directly on NOX. The broken-cell assay allows both better specification of the source of the ROS, and even the NOX isoform, as well as distinction between a direct inhibitor and an inhibitor of the signaling pathway leading to NOX activation. Because of the highly hydrophobic nature of the NOX transmembrane domains, a completely recombinant assay is not yet feasible. Therefore, this approach currently requires purification of the transmembrane component(s) of the enzyme and addition of either cytosol, recombinant cytosolic factors, or chimeras thereof [for review, see (23)]. Inclusion of recombinant forms of cytosolic factors that are in a constitutively active state permits the detection of direct inhibitors. Researchers often choose to measure the oxidation of the NADPH substrate or the diaphorase activity of the enzyme by using iodonitrotetrazolium (INT) violet as electron acceptor, instead of measuring the reaction product (superoxide), because this approach eliminates potential interference by ROS scavenging (43). However, this technique is still a matter of debate because its specificity is questionable, as INT has been shown to react preferentially with the hemes rather than with the FAD redox center of flavocytochrome and is not, therefore, a specific probe of the diaphorase activity of flavocytochrome (86).

Measurement of direct binding. Radiolabeled compounds such as DPI (21, 38) and phenylarsine oxide (29) were used in a photoaffinity approach to demonstrate direct binding to NOX2.

### How Can a Compound Inhibit the Oxidative Burst without Affecting NOX Enzymes Directly?

Many compounds decrease NOX-generated ROS in a variety of cell types. However, one should be aware that blunting any step of the NOX activation process can decrease NOX activity. Some of these off-target effects are summarized later. Compounds that act at these levels are not considered further in this review.

#### *Inhibition of the activation mechanism*

A compound may interfere upstream at the level of activation, for example, by blocking the receptors for formyl-methionyl-leucyl-phenylalanine (fMLF), a physiologic peptidic activator of NOX2, or by blocking the receptor for angiotensin II, an activator of NOX1 (62). It is therefore useful to test different activating agents when assessing the efficiency of a NOX inhibitor. Similarly, agents that block calcium channels may prevent the activation of NOX5 and DUOX, but cannot be considered true NOX inhibitors.

#### *Signal transduction*

Compounds may act farther along the activation pathway, by affecting phosphorylation through effects on protein kinases, phospholipases, phosphatases, or G proteins. Blocking phosphorylation of $p47^{phox}$ and preventing guanine nucleotide exchange on Rac both interfere with NOX activation (24).

#### *Second messengers*

Agents that limit the availability of $Ca^{2+}$ can inhibit NOX2 activation (58), whereas compounds that increase cAMP levels inhibit NOX2 by acting on upstream signaling (45, 69).

*Electron-donor availability*

Compounds that decrease the cytosolic levels of NADPH, such as inhibitors of the pentose phosphate pathway, can preclude NOX activation (36, 37).

*Endogeneous antioxidant systems of the cell*

Some compounds have been shown to upregulate endogenous cellular defense against ROS, such as superoxide dismutases (109), glutathione (32), or thioredoxin (102).

Typical examples of indirect NOX inhibitors include angiotensin AT1-receptor blocker, such as valsartan (119), statins [3-hydroxyl-3-methylglutaryl coenzyme A (HMG-CoA) reductase inhibitors] through inhibition of geranylgeranylation of Rac (17, 71), nebivolol (78), mycophenolate by decreasing NOX expression (54, 61), dextromethorphan, and related opioid compounds, which protect neurons by inhibiting microglial activation through a NOX2-dependent mechanism (12, 64, 121, 124).

## List of NOX Inhibitors

Hundreds of compounds have been shown to decrease ROS generation in neutrophils, vascular cells, or other systems wherein NOX enzymes are the principal source of ROS. It was not the purpose of this review to make an exhaustive list, but rather to focus on (a) small molecules for which substantial evidence exists for NOX inhibition, according to the flow chart described earlier; and (b) compounds from the patent literature that are likely to have been selected according to their potential to become a useful drug. Comparing the structural features of compounds with a direct effect on the NOX system might be expected to reveal common motifs indicative of specific binding pockets in the NOX complex.

*Direct inhibitors of the NOX catalytic subunit*

The following list encompasses the inhibitors for which most required experimental data showing NOX inhibition have been published. Their characterization includes (a) measurement of ROS production, (b) oxygen consumption, (c) viability, (d) exclusion of a predominant scavenging effect, and (e) NOX inhibition in a broken-cell assay (Table 1). The chemical structures of these compounds are represented in Fig. 2.

Aryliodonium compounds. Diphenyleneiodonium (DPI) has been used extensively as a NOX inhibitor, but other organic iodine compounds are also effective, likely based on a similar mechanism (28). The $IC_{50}$ for inhibition of neutrophil NOX2 activity by iodonium diphenyl is 80 $\mu M$ (38), whereas other iodine inhibitors are more potent to inhibit neutrophil NADPH oxidase activity with an $IC_{50}$ of 0.5 $\mu M$ for di-2-thienyliodonium, and an $IC_{50}$ of 0.75 $\mu M$ for phenoxaiodonium (70).

DPI. DPI, which inhibits all NOX and DUOX isoforms at low micromolar to submicromolar concentrations has been widely used to provide evidence for the presence of NOX activity in tissue (9, 25, 80). The mechanism of action has been studied in detail. DPI is thought to act by abstracting an electron from FAD to form a radical, which then forms covalent adducts binding irreversibly to the FAD of NOX enzymes, blocking their activity rapidly and irreversibly (76). However, DPI is nonspecific (77), inhibiting a number of other enzymes (5, 92), some with greater potency [*e.g.*, $IC_{50} < 100$ n$M$ for NOS (100)]. Nevertheless, it remains useful if experiments are well controlled. As a rule of thumb, in a cellular assay the absence of inhibition of ROS after a short preincubation (<30 min) with a low concentration of DPI (<10 $\mu M$) provides convincing evidence against NOX as the source of ROS. Long-term exposure to DPI leads to a wide range of nonspecific effects, as reviewed in (5).

DPI is a useful tool for studying NOX enzymes *in vitro*, but it is not a drug candidate, because of irreversible binding, off-target effects, and low solubility. Moreover, it exhibits high toxicity in rodents with a $LD_{50} < 10$ mg/kg (33). Hypoglycemia is an acute adverse effect of DPI (42), whereas prolonged administration (1.5 mg/kg/d over a 4- to 5-week period) induced cardiomyopathy (19). However, low-dose DPI has been used for target validation of NOX-dependent pathology *in vivo*. In a model of hemorrhagic shock, administration of 1 mg/kg IV resulted in a decrease in superoxide anion production measured in liver by lucigenin-enhanced chemiluminescence, as well as attenuation of lung and intestinal injury (1). In a rat model of vasospasm induced by subarachnoid hemorrhage (SAH), 5 $\mu$g/kg DPI injected directly into the cisterna magna led to a decrease in lucigenin chemiluminescence in the cerebral vasculature and mitigated SAH-induced changes in the luminal perimeter of the middle cerebral artery (50). Vlessis *et al.* (109) measured total-body oxygen consumption in a metabolic chamber after phorbol myristate acetate (PMA) challenge (109). The increase in oxygen consumption, thought to be the result of the phagocyte respiratory burst, was blunted by DPI (3.5 mg/kg IV) (109). This treatment protected against PMA-induced lung injury. The prolonged administration of low doses of DPI (0.5, 5, 10, or 50 $\mu$mol/kg/d IP, corresponding to $\sim$0.15–15 $\mu$g/kg) to mice nearly abolished potassium peroxochromate–induced arthritis and whole-blood lucigenin chemiluminescence (68). More surprisingly, 1 mg/kg SC for 4 weeks dramatically protected against alcohol-induced liver injury in the rat and diminished by 90% the detection by ESR of free radical adducts in the bile (53).

Thiol-modifying compounds. Sulfhydryl-modifying reagents such *N*-ethylmaleimide (NEM) or *p*-chloromercuribenzoate have long been known to prevent activation or assembly of NOX2 (3, 20).

Phenylarsine oxide (PAO). PAO is a potent NOX2 inhibitor, but its potential effects on other NOX isoforms have not yet been determined. PAO at 1 $\mu M$ completely blocks NOX2 activation in the neutrophil without affecting other cellular functions, such as phagocytosis and degranulation (63). Its particular mode of action has been studied in detail: (a) PAO is ineffective once the oxidase complex is formed, suggesting that it prevents assembly of an active oxidase complex or that assembly blocks access to the PAO-binding site; and (b) the inhibitory action of PAO can be reversed by a vicinal dithiol competitor 2,3-dimercaptopropanol, but not by mercaptoethanol (56). As PAO is known to interact with vicinal cysteine residues, it is postulated that in the resting state the NOX subunit contains unmasked PAO-accessible SH groups belonging to vicinal or neighboring Cys residues and that these SH groups are no longer accessible once NOX2 is in

Table 1. Molecules with Documented Direct Effect on NOX Enzymes in Cell-free Systems

| *Compounds* | *Reactive oxygen production* | *$O_2$ consumption* | *Viability* | *Scavenging* | *Membrane assay* | *Other* |
|---|---|---|---|---|---|---|
| Diphenylene-iodonium (21, 38, 94, 95) | $IC_{50} = 0.90\ \mu M$ for neutrophil NOX2 activity.<br>All NOX enzymes are inhibited in the submicromolar range:<br>NOX4: $IC_{50} = 0.2\ \mu M$<br>NOX5: $IC_{50} =$ 0.1–0.3 $\mu M$ | Complete inhibition at $<10\ \mu M$ | No cell toxicity at efficient concentration | Inhibits completely X/XO, but no scavenging activity | NOX2<br>NOX4 | Inhibitors of all flavin-containing proteins and other nonspecific actions are reviewed in (5) |
| Phenylarsine oxide (3, 29, 63) | $IC_{50} = 0.5\ \mu M$ in PMA-activated neutrophils | ND | No cell toxicity after 30-min incubation | ND | Completely blocks at $1\ \mu M$; forms disulfide bond with vicinal cysteinyl residues | No inhibition of protein kinases at concentrations used |
| Gomisin C (112) | fMLF-activated neutrophils: $IC_{50} = 21.5\ \mu g/ml$ | fMLF-activated neutrophils $IC_{50} = 26\ \mu g/ml$ | ND | No superoxide scavenging | 40% at $30\ \mu g/ml$ | Slight inhibition of $Ca^{2+}$ entry |
| Abruquinone A (43) | fMLF-activated neutrophils: $IC_{50} = 0.33\ \mu g/ml$<br>PMA-activated neutrophils: $IC_{50} = 0.49\ \mu g/ml$ | fMLF- or PMA§-activated neutrophils: ~50% inhibition at $3\ \mu g/ml$ (irreversible) | >95% viability at $10\ \mu g/ml$ for 3 min | No superoxide scavenging | $IC_{50} = 0.6\ \mu g/ml$<br>$IC_{50} = 1.5\ \mu g/ml$ for diaphorase activity | Inhibition of inositol trisphosphates 2 and 3 ($IC_{50} = 10\ \mu g/ml$)<br>Inhibition of $Ca^{2+}$, $IC_{50} =$ $7.8\ \mu g/ml$ |
| Norathyriol (44) | fMLF-activated neutrophils: $IC_{50} = 6.9\ \mu M$ | fMLF-activated neutrophils: ~50% inhibition at $30\ \mu M$ (reversible) | ND | DHF: $IC_{50} = 15\ \mu M$<br>X/XO $IC_{50} = 2.5\ \mu M$, but also blocks uric acid formation (60% at $30\ \mu M$) | $IC_{50} = 18\ \mu M$<br>Noncompetitive for NADPH | Blockade of phospholipase C and protein tyrosine phosphorylation |

| | | | | | | |
|---|---|---|---|---|---|---|
| Magnolol (111) | Whole-blood assay 30 mg/kg IP Rat neutrophils: $IC_{50} = 15.4\ \mu M$ | Efficiently blocks $O_2$ consumption | 95% viability at 100 $\mu M$ | No superoxide scavenging | 26.7% inhibition at 100 $\mu M$ | Blockade of different protein kinases |
| Neopterin (52) | $IC_{50} = 1–2\ \mu M$ in PMA-activated rodent macrophages | ND | ND | Low superoxide scavenging activity, $IC_{50} = 370\ \mu M$ | $IC_{50} = 1.23\ \mu M$ Competitive for NADPH: apparent $Ki = 6.5\ \mu M$ | Neopterin is present in body fluids, and its elevation is a marker of cell-mediated immunity |
| Gliotoxin (94, 106, 122) | PMA-activated neutrophils: $IC_{50} = 2.95–11\ \mu g/ml$ NOX4: $IC_{50} > 100\ \mu M$ | ND | No toxicity on neutrophils | ND | $IC_{50} = 3.3\ \mu M$ | Blocks NOX only before it is activated by reacting with thiol residues |
| Perhexiline (49) | PMA-activated neutrophils: $IC_{50} = 2.3\ \mu M$ | ND | ND | ND | $IC_{50} = 26.2\ \mu M$ in neutrophils $IC_{50} = 2.7\ \mu M$ in HUVECs. $IC_{50} = 7.5\ \mu M$ in valve interstitial cells $IC_{50} = 6.4\ \mu M$ in cardiac fibroblasts | Does not affect subunit preassembly No inhibition of ROS production by rat aortic rings and aortas up to 20 $\mu M$ (NOX1 and NOX4) |
| Prodigiosin (73) | PMA-activated mouse macrophage cell line: $IC_{50} = 6.9\ \mu M$ | ND | ND | No superoxide scavenging (X/XO) | 1 $\mu M$ blocks 80% activity in reconstituted system | Binds to the membrane and inhibits the association between rac and $p47^{phox}$ No inhibition of protein kinase C |
| Honokiol (65) | fMLF and PMA-activated neutrophils: $IC_{50}$ = approx 10 $\mu M$ | ND | 97% viability at 10 $\mu M$ | ND | Blocks completely superoxide production at 1 $\mu M$ after enzyme is activated, but not before | ND |

X/XO, xanthine/xanthine oxidase; ND, not documented; fMLF, formyl-methionyl-leucyl-phenylalanine; PMA, phorbol 12-myristate 13-acetate; DHF, dihydrofumaric acid; NADPH, reduced nicotinamide adenine dinucleotide phosphate; HUVECs, human umbilical vein embryonic cells.

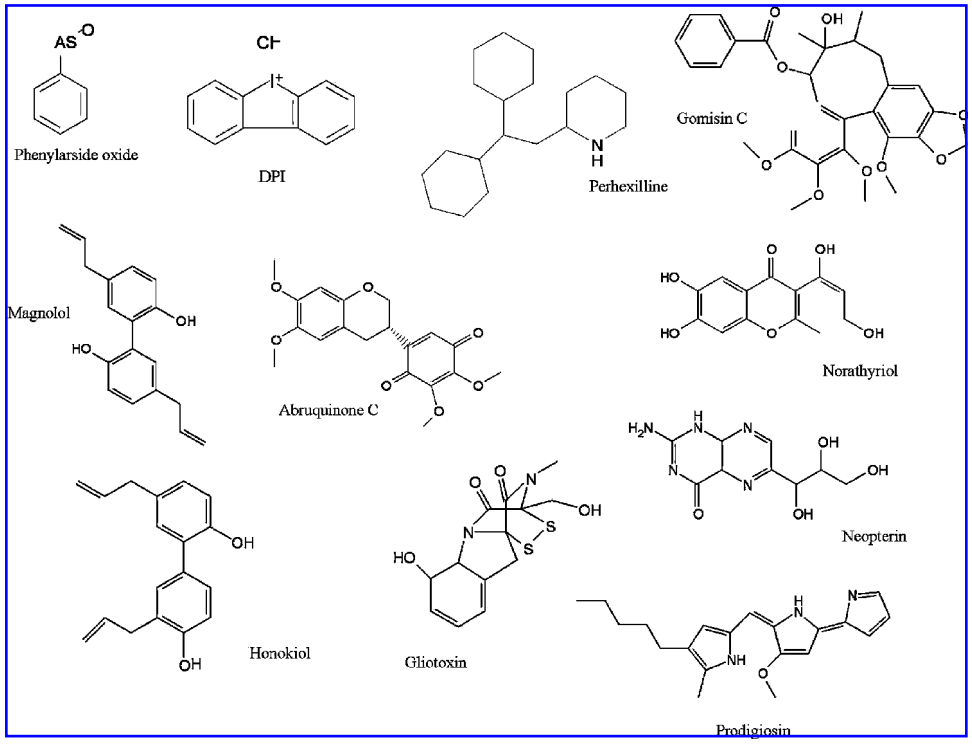


**FIG. 2. Two-dimensional structures of NOX inhibitors having a direct effect on the catalytic core of NOX enzymes.**

its activated state (29). This postulate would make PAO a specific NOX2 inhibitor, because NOX2 is the only isoform containing two neighboring Cys residues (positions 368 and 370), but this prediction has not yet been validated. However, this general chemical reactivity with reactive cysteine residues is likely to make this agent an inhibitor of a variety of other enzymes and proteins and therefore inappropriate as a specific probe or drug candidate. Nevertheless, experiments using PAO *in vivo* provide hints as to the possible utility of NOX-directed specific inhibitors. For example, injection of PAO (1 mg/kg IP) had an antiinflammatory action on both hind paw edema induced by carrageenan and lung inflammation induced by lipopolysaccharide inhalation, and was able to decrease ROS production elicited by opsonized zymosan in whole blood (90).

Gliotoxin (GTX). GTX is a disulfide-containing mycotoxin extracted from *Aspergillus* species. It blocks NOX2 in neutrophils ($IC_{50} = 8\,\mu M$) (122), but it is rather inefficient with respect to NOX4 (94). Although it has been shown that GTX inhibits the neutrophil oxidative burst by interfering with $p47^{phox}$ phosphorylation (106), it also directly affects NOX2 in both intact cells and a semirecombinant cell-free assay ($IC_{50} = 3.3\,\mu M$) (75). It is speculated that GTX blocks NOX2 activity by a mechanism similar to that of PAO, because (a) GTX inhibits the electron transport of NOX2 only before but not after oxidase activation; and (b) inhibition of NOX2 is completely abolished by simultaneous addition of the reducing agent dithiothreitol or when the *S*-methylated form of GTX is used instead of GTX. This inhibitory action of GTX has been suggested to contribute to the pathogenicity of *Aspergillus* species by suppressing innate host defenses (75). In addition, at lower (submicromolar) concentrations, GTX was shown to inhibit neutrophil phagocytosis without affecting the oxidative burst, and to induce reorganization of the actin cytoskeleton, leading to shrinkage, probably by modifying intracellular cAMP homeostasis (18).

### *Natural compounds from plants*

Natural compounds from plant extracts with antiinflammatory properties tend to show a large and complex panel of effects that contribute to depression of the immune response, including in some cases inhibition of NOX2. None of these compounds has been tested against other NOX isoenzymes. A number of natural compounds were systematically tested for their inhibitory effect on the oxidative burst. The compounds described later are the only ones that also showed an inhibitory action in a cell membrane assay consisting of particulate NOX2 plus cytosol. Unfortunately, although their effect on the oxidative burst has been studied, little is known about their effects on other enzymatic systems.

Norathyriol. Norathyriol is a xanthone aglycon isolated from *Tripterospermum lanceolatum* with radical scavenging properties and NOX2 inhibitory action. Norathyriol inhibits INT reduction by the arachidonate-activated phagocyte cell-free NADPH oxidase system, as well as NADPH oxidase activity in membranes from PMA-activated neutrophils, both with $IC_{50}$ values around 18 $\mu M$ (44). However, norathyriol has many other effects, such as inhibition of xanthine oxidase, ROS scavenging, blockade of the phospholipase (PL) C pathway, reduction of protein tyrosine phosphorylation (44), and protein kinase (PK) C modulation (114); for a review, see ref. 85.

Gomisin C. Gomisin C is a lignan extracted from *Schizandra chinensis* with a weak inhibitory action on NOX2. It also decreases cytosolic $Ca^{2+}$ release from intracellular stores (112). No *in vivo* data are available with this compound.

Abruquinone A. Abruquinone A is a natural isoflavanquinone isolated from the roots of *Abrus precatorius.* Like norathyriol, abruquinone A is able to block NOX2-dependent ROS generation and diaphorase activity in particulate fractions and intact neutrophils with an $IC_{50}$ around 1 $\mu M$ (43). Direct NOX2 inhibition is considered to be the main site for oxidative burst inhibition, although this effect may also be partly attributable to blockade of PLC and PLD pathways (43). No *in vivo* data are available with this compound, but its effects on other NOX isoforms might be informative.

Magnolol. Magnolol is a hydroxylated biphenyl compound isolated from the Chinese herb *Magnolia officinalis*. In addition to its inhibitory effect on isolated neutrophils ($IC_{50} \approx 15.4\ \mu M$) and particulate NADPH oxidase at higher concentration (100 $\mu M$), this compound inhibits lucigenin chemiluminescence of neutrophils in whole blood after injection (30 mg/kg IP) (111). Magnolol shows a number of pharmacologic properties including a proapoptotic effect (16, 46) and binding to GABA receptors (2), but it has not been tested on other NOX isoforms.

Honokiol. Honokiol is an active component isolated from the herb *Magnolia officinalis*. Honokiol has many antioxidant properties and has been shown to have activity *in vivo*, such as reducing myocardial infarct size and cardiac arrhythmias in rats after coronary artery occlusion (105) and mitigating brain ischemia/reperfusion injury in a rat model (66). In neutrophils, honokiol was able to block the oxidative burst in both intact cells and particulate fractions (65). Although this compound seems to have a broad array of pharmacologic properties, it is interesting because it completely blocks superoxide production at 1 $\mu M$ after the enzyme is activated, but not before, and would therefore be potentially useful in pathologic conditions when the enzyme is persistently activated. Low concentrations of honokiol (0.125–1 $\mu M$) suppress apoptosis in high glucose–induced human umbilical vein endothelial cells (HUVEC) through NOX inhibition (96).

Prodigiosin. Prodigiosins are natural red pigments with numerous pharmacologic activities. They are immunosuppressants with anticancer activity (83). In neutrophils, prodigiosin was shown to inhibit NOX2 through binding to the membrane and inhibiting the association between Rac and $p47^{phox}$, without affecting PKC activity (73).

### *Endogeneous compounds*

Neopterin. Neopterin, a breakdown product of GTP, is synthesized by activated macrophages. The compound inhibits NOX2 in both intact macrophages ($IC_{50} = 1.4\ \mu M$) and a cell-free NOX2 system ($IC_{50} = 1.2\ \mu M$). Of particular interest is the fact that a Lineweaver–Burke plot showed that the inhibition of particulate macrophage NOX2 by neopterin was competitive with respect to NADPH (52). Neopterin is present in body fluids, and its elevation is a marker of cell-mediated immune responses (11); although its physiologic function is not understood, it is tempting to speculate that neopterin is a physiologic inhibitor of NOX2, and that it functions to prevent excess ROS production and resulting tissue damage in inflammatory states (for review, see ref. 24).

### *Synthesized compounds*

Perhexiline. Perhexiline is a prophylactic antianginal agent that is used clinically when other agents are ineffective. The fact that it blocks superoxide generation by neutrophils, HUVECs, cardiac valve interstitial cells, and cardiac fibroblasts, but not aortic tissues, speaks against ROS scavenging and suggests some degree of isoform selectivity (49).

## Indirect Inhibitor of the NOX Catalytic Subunit

### *Apocynin*

Although controversial, substantial evidence shows an inhibitory activity of apocynin on NOX2 (Table 2). The chemical structure of this compound is represented in Fig. 3. Apocynin has not been shown to act directly at the level of the catalytic flavocytochrome, and it has not been tested according to the flow chart in Fig. 1. In particular, it has not been shown to block the enzyme directly in a cell-free assay. Nevertheless, evidence suggests that, under appropriate conditions, apocynin acts as an NOX inhibitor.

Apocynin is a natural methoxy-substituted catechol isolated from *Picrorhiza kurroa*, which has long been used as an NOX inhibitor. Its use has been extensively described, as it is a very efficient substance for decreasing the symptoms in animal models in which oxidative stress is involved, such as hypertension, atherosclerosis, and stroke (for review, see refs. 92, 97). However, part of the controversy surrounding the use of apocynin comes from the fact that it combines an inhibitory effect on NOX under certain conditions with ROS-scavenging activity (40, 104). The controversy is ongoing, and apocynin has been the subject of several recent reviews (5, 97, 117). Detailed descriptions of the numerous studies showing an effect of apocynin in ROS-mediated models is beyond the scope of this review.

Nevertheless, the intriguing effects of apocynin on NOX enzymes can be summarized as follows:

1. The most striking effect of apocynin is its high potency in suppressing symptoms in *in vivo* pathologic models after prolonged administration. For example, in a model of salt-loaded stroke-prone spontaneously hypertensive rats, apocynin (0.6 mmol/kg/day for 4 weeks) prevented the occurrence of stroke (119). In a transgenic mouse model of amyotrophic lateral sclerosis ($hSOD1^{G93A}$), apocynin improved survival in a dose-dependent manner up to more than twice with the highest dose used (300 mg/kg/day) (39). Therefore, high doses of apocynin can be administered in the drinking water for weeks without showing adverse effects. Prolonged *in vivo* treatment with apocynin resulted in a positive outcome in a whole range of pathologies in which oxidative stress is involved, including diabetic nephropathy (40), cardiac hypertrophy, aneurysm formation (6), or retinal vascular inflammation (4), among others.
2. Short-term administration of apocynin is also efficient. Inhalation of apocynin mitigated ozone-induced airway hyperresponsiveness and no adverse effects in asthmatic subjects (3 ml of 0.5 mg/ml was inhaled 2 times before and 6 times after ozone exposure at hourly intervals) (84). Single doses of apocynin in liver ischemia/reperfusion (3 mg/kg IP) (67) or transient middle

Table 2. Molecules with No Documented Effect in Cell-free Assays, but Strong Evidence on NOX Inhibition

| *Compounds* | *Reactive oxygen production* | *$O_2$ consumption* | *Viability* | *Scavenging* | *Membrane assay* | *Other* |
|---|---|---|---|---|---|---|
| Apocynin (40, 99, 107) | $IC_{50} = 10\,\mu M$ in opsonized zymosan-stimulated neutrophils or in assays using HRP<br>$IC_{50} > 1{,}500\,\mu M$ in PMA-stimulated neutrophils (HRP-free assay) | $300\,\mu M$ completely inhibits opsonized zymosan-stimulated neutrophils | No toxicity | ~50% scavenging at $100\,\mu M$ | ND | Nonspecific actions are reviewed in (5) |

HRP, horseradish peroxidase; PMA, phorbol 12-myristate 13-acetate; ND, not documented.

cerebral artery occlusion (2.5 mg/kg IV) (101) showed great efficacy at reducing symptoms and pathologic end points. Apocynin acts as radical scavenger at concentrations $>100\,\mu M$ (40), but can also inhibit NOX activity ($IC_{50} = 10\,\mu M$) in neutrophils under specific conditions (107), such as stimulation by opsonized zymosan (but not by PMA) and after a lag time.

3. Updates of what is known about the pharmacology of apocynin were recently discussed in detail (5, 92, 97). The pharmacology of apocynin is quite complex because, to exhibit efficient direct inhibition of NOX, it is thought to act as a pro-drug, requiring both hydrogen peroxide and a peroxidase—in particular myeloperoxidase (MPO)—to promote its dimerization to an apocynin radical, which then oxidizes thiols in the NADPH oxidase protein components (99). Because neutrophils and other phagocytic cells have both a hydrogen peroxide–generating system (NOX2) and MPO, apocynin can be converted into the active dimer in these cells, but not in cells lacking these systems, such as fibroblasts (108), vascular smooth muscle cells, or transfected human embryonic kidney cells (40). In nonphagocytic cells, apocynin (30–$300\,\mu M$) has also been shown to increase ROS production (88, 108). However, a recent study evaluated the bioavailability of apocynin *in vivo* and seriously challenged this theory for the *in vivo* pharmacologic action of apocynin. After IP administration, ~50% of apocynin was converted to its glycoconjugate, but no diapocynin was detected. However, the glycoconjugate derivative of apocynin was detected in plasma, liver, and brain (116). The activity of this derivative has not yet been tested for scavenging or NOX inhibition.
4. Apocynin (or at least its active radical) is considered to act through inhibition of interaction of the $p47^{phox}$ subunit with the catalytic part of NOX2 (8, 47, 99). If this mechanism is correct, apocynin should be a specific inhibitor of NOX isoforms that depend on cytosolic subunits (*i.e.*, NOX1, NOX2, and NOX3), but not NOX4, NOX5 (40, 94, 95), and DUOX1 and 2, but to our knowledge, the latter has not been tested.

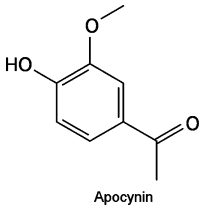


**FIG. 3. Two-dimensional structure of apocynin.**

In conclusion, apocynin has not been shown to be active in a cell-free assay, as it probably interferes with the assembly of the oxidase under conditions found only in the intact cell. Its use in cellular assays to inhibit NOX at concentrations $>100\,\mu M$ is not recommended, because it acts as a radical scavenger at this concentration. *In vivo*, apocynin is obviously a potent antioxidant drug with good bioavailability, including blood–brain barrier permeability. However, it is still unclear which parts of this strong antioxidant effect are mediated by radical-scavenging effect *versus* NOX inhibition.

## NOX Inhibitors Developed by Pharmaceutical Companies and Other Patented Inhibitors

The following list encompasses the inhibitors specifically developed by the pharmaceutical industry. (Table 3). Their chemical structures are represented in Fig. 4.

For this list of compounds, inhibition of NOX activity was discovered largely as a result of investigations into the pharmacologic mechanisms of action of known compounds with previously documented mechanisms of action. A more directed approach has been taken largely by pharmaceutical companies to identify NOX inhibitors, based, for example, on high-throughput screening of chemical libraries. Recent interest by companies in developing NOX inhibitors has led to the publication of some scientific articles and patent applications. In many cases, information available through patents is incomplete or cryptic, and much information is proprietary, but no doubt, some of these compounds have interesting properties and may be useful as a starting point for drug development.

Table 3. Molecules Discovered by Pharmaceutical Companies

| *Compounds* | *Reactive oxygen production* | *Membrane assay* | *Other* |
|---|---|---|---|
| VAS2870: 3-benzyl-7-(2-benzoxazolyl) thio-1,2,3-triazolo [4,5-d]pyrimidine (Vasopharm) (98, 103) | $IC_{50} = 2\ \mu M$ in PMA-stimulated oxidative burst in HL-60 (human leukocyte cell line) and vascular cells | $10\ \mu M$ in membrane assay | ND |
| *N*-(1-cyclohexylethyl)-4-phenylphthalazin-1-amine (Mitsubishi) (120) | Inhibition of superoxide production in diabetic aorta | ND | $6.3\ nM = IC_{50}$ of IL-8 production in HUVECs stimulated with high glucose (NOX-dependent); inhibition of atherosclerosis; protective in ischemia/reperfusion experiments in brain and heart |
| Pyrazolo (1.5-A) pyrimidines (Shionogi) (93) | ND | Exhibit $IC_{50} < 1\ \mu M$ in bovine aortic membrane fractions | *In vivo* efficacy on neutrophils and blood vessels |
| S17384 (Servier) (15, 48, 123) | 50% inhibition between 25 and $50\ \mu M$ in HUVECs after tumor necrosis factor-α incubation | 50% inhibition between 25 and $50\ \mu M$ in endothelial cell fractions after tumor necrosis factor-α incubation | Decrease of atherosclerotic lesions *in vivo* (130 mg/kg/day for 12 wk) in ApoE-deficient mice fed with chow and in streptozotocin-induced diabetes in LDL receptor–deficient mouse (6 wk) |
| Pyrazolo pyridines (Genkyotex) (81) | ND | $IC_{50} = 1\text{-}10\ \mu M$ on all NOX isoforms | ND |
| Tetrahydroindoles (Genkyotex) (82) | ND | $IC_{50} = 1\text{-}10\ \mu M$ on all NOX isoforms | ND |

PMA, phorbol 12-myristate 13-acetate, ND, not documented; HUVECs, human umbilical vein embryonic cells.

### *VAS2870 (Vasopharm)*

With PMA-stimulated neutrophil-like HL-60 cells, VAS2870 had an $IC_{50}$ of $2\ \mu M$ on NOX2 and $10.6\ \mu M$ in a cell-free NOX2 system consisting of plasma membrane and cytosol from human neutrophils (98, 103). At $10\ \mu M$, this compound inhibited oxidized low-density lipoprotein–induced ROS formation in endothelial cells and platelet-derived growth factor–stimulated ROS generation in vascular smooth muscle (98, 103). In addition, VAS2870 ($50\ \mu M$) significantly inhibited the vasculogenesis of embryonic stem cells after treatment with platelet-derived growth factor BB (60). However, no *in vivo* data are available for this compound.

### *S17834 [6,8-diallyl 5,7-dihydroxy 2-(2-allyl 3 hydroxy 4-methoxyphenyl) 1-H benzo (b) pyran-4-one] (Servier)*

S17834 was shown to inhibit superoxide production in HUVECs (15, 48). S17834 inhibited both superoxide production by intact HUVECs and NADPH consumption by endothelial cell membrane fractions after 2- and 4-h treatment, respectively, with TNF-α (50% inhibition between 25 and $50\ \mu M$) without affecting superoxide production by the xanthine/xanthine oxidase system (15). *In vivo* administration of 130 mg/kg/day S17834 for 12 weeks to ApoE-deficient mice fed with chow to induce atherosclerosis led to a significant reduction in lesion development compared with that in untreated mice, as well as a decrease of superoxide generation in the aorta (15). In another *in vivo* model of streptozotocin-induced diabetes in LDL receptor–deficient mice, 6 weeks of treatment with a similar dose of S17834 inhibited aortic atherosclerotic lesion development (123).

### *N-(1-cyclohexylethyl)-4-phenylphthalazin-1-amine (Mitsubishi)*

In the patent describing this compound, NOX inhibitory action is shown in an unusual assay. This compound was shown to have an $IC_{50} = 6.3\ nM$ against NOX-dependent IL-8 production in HUVECs stimulated with high glucose; in addition, the claim was made that these compounds showed no inhibitory effect on NOX2 from leukocytes, but only on the excessive effect of NOX in other tissues, such as inhibition of superoxide production in diabetic aorta. Use of this compound *in vivo* protected from atherosclerosis and ischemia/reperfusion damage in brain and heart (120).

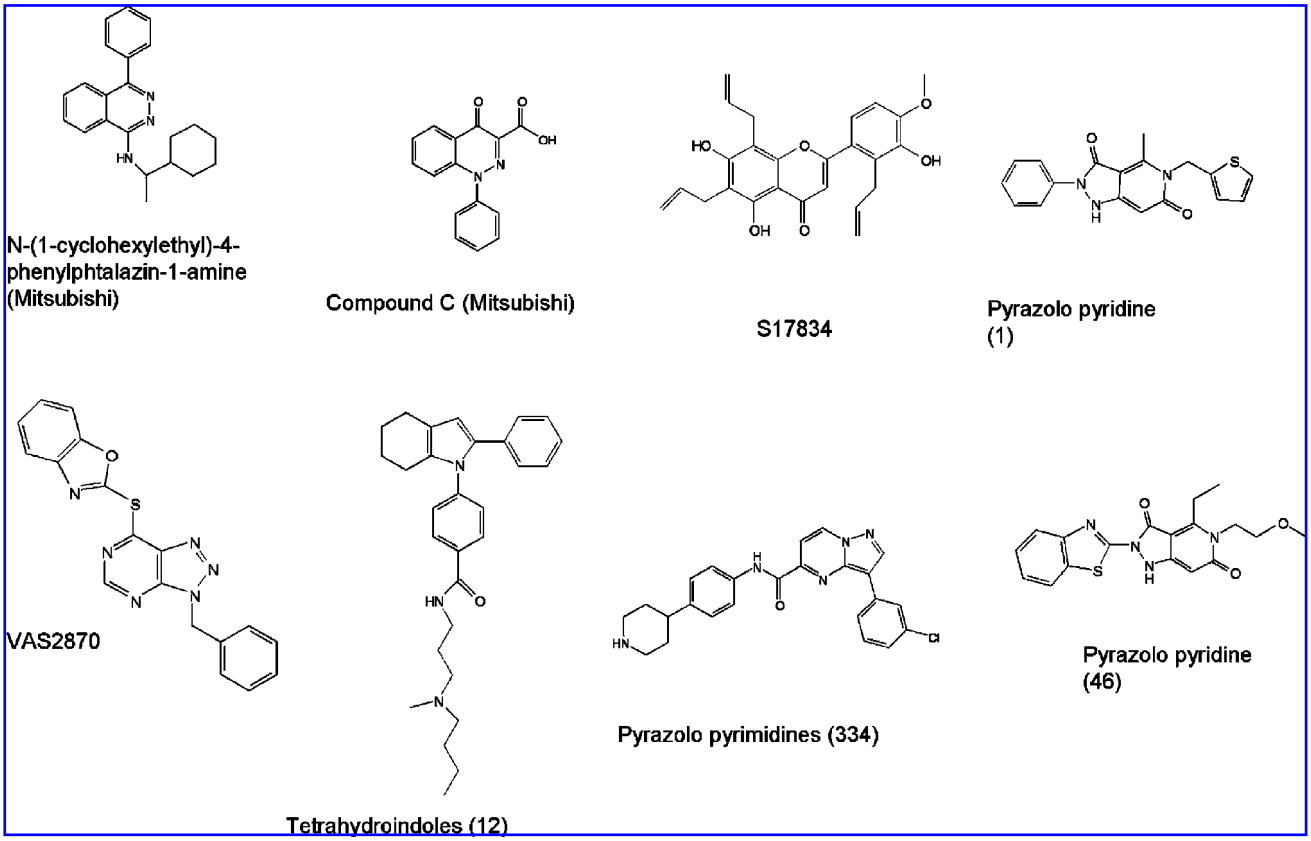


**FIG. 4. Two-dimensional structures of NOX inhibitors developed by pharmaceutical companies.** The compounds depicted for the pyrazolo pyrimidine, tetrahydroindole and pyrazolo pyridine families are those reported in the patent having the highest activities. Bracketed numbers correspond to the number of the compound within the corresponding patent.

*Pyrazolo (1.5-A) pyrimidines (Shionogi)*

Little public information of the biologic activity is provided for these compounds. However, it is claimed in the patent application that a number of similar compounds exhibit $IC_{50} < 1\,\mu M$ in inhibiting NADPH oxidase in bovine aortic membrane fractions, as well as *in vivo* efficacy on neutrophils and blood vessels (93).

*Tetrahydroindole derivatives (GenKyoTex)*

NOX inhibitory activity for these compounds is documented as being in the low micromolar range. Other public information on their biologic activity is not available (82).

*Pyrazolo pyridines (GenKyoTex)*

Very little public information on the biologic activity is available for these compounds, although they are claimed to exhibit NOX inhibitory activity in the low micromolar range (81).

## How Similar/Diverse Are the Reported Chemical Compounds?

The most striking feature of all chemical structures described in this review (with the exception of perhexiline) is the extended double-bond conjugated systems, which are able to mediate electron exchange important in redox reactions, such as those involving ROS production.

To evaluate the structural diversity/similarity of the compounds, the *Tanimoto coefficient* (TC) was calculated. The TC is an association coefficient used to determine chemical similarity, which can range from 0, indicating no similarity, to 1, suggesting that the compounds are identical (34, 74). In addition, all compounds were compared with the cofactor FAD and substrate NADPH (Fig. 5). For all NOX inhibitors described, the calculated TCs are low, with the exception of magnolol and honokiol (TC = 0.92), which differ only on the position of the 1-hydroxyl group on the phenyl ring, FAD and NADPH (TC = 0.57), which have in common the adenosine moiety and, to a lesser extent, gomisin C and abruquinone A (TC = 0.3). Therefore, compounds in Table 1 can be classified as structurally very diverse from each other. No similarity was found either between compounds of Table 1 and the molecules of Tables 2 and 3 except for magnolol and honokiol sharing some low level of similarity with apocynin (TC = ca. 0.3). Compounds from Table 2 share no similarity with FAD or NADPH as TC < 0.1, whereas all compounds from Table 3 share some similarity to FAD and NADPH (TC ranging from 0.25 to 0.3), suggesting a mode of action related to NADPH- and FAD-binding sites.

In conclusion, molecules of Tables 1 and 2 cover a wider range of the chemical space compared with compounds of

Table 3 because of the enhanced structural diversity. Unfortunately, as the 3D structure has not yet been solved for NOX enzymes, only hypotheses can be made: assuming that all the presented NOX inhibitors have a direct effect on NOX enzymes, the large structural diversity of these molecules is indicative of a large binding pocket, allowing a wide range of molecules to interact or discrete binding sites. Such a large binding pocket has been described for the cytochrome P450 enzymes, which also contains a catalytically essential heme group in which a large number of molecules are known to interact, even at remote locations (7).

However, because of the heterogeneity of the biologic data and the chemical diversity of the presented compounds, a structure–activity relation is virtually impossible.

## Conclusions and Perspectives

Based on the available scientific literature, in particular on studies with NOX-deficient mice, it is increasingly clear that inhibition of NOX enzymes is a promising pharmacologic concept for oxidative stress–mediated diseases. As opposed to antioxidants, including ROS scavengers, whose action maybe summarized as "too late, too little, too nonspecific," selective or specific NOX inhibitors would directly block the production of ROS, rather than trying to mitigate the effects of ROS that have already been produced. Also, scavenging mechanisms are easily overpowered by high concentrations of the compounds to be scavenged and hence are least efficient at the site of pathologic overproduction. Such concentration-dependent loss of efficacy does not apply to enzyme inhibitors. Similarly, by developing NOX isoform–specific inhibitors, a high degree of specificity could be reached, something impossible to achieve with ROS scavengers.

However, despite this promise, there is still a long way to go. To date, most NOX inhibitors described in the literature either have been incompletely characterized or show numerous other pharmacologic properties or lack desirable druglike qualities. Moreover, on prolonged administration, a possible side effect of NOX2 inhibition would be decreasing the ability of the innate immune system to destroy some microorganisms and/or the development of local hyperinflammatory states (91). The complete loss of NOX2 function results in chronic granulomatous disease (CGD), a hereditary disease characterized by the development of granulomas, particularly in the colon, and by a susceptibility to certain fungal and bacterial infections due to impaired killing of microorganisms (91). This is certainly a concern for a use of NOX inhibitors in humans. However, CGD carriers in which only 5–10% of neutrophils generate ROS do not have obvious symptoms (55). To date, no specific additional abnormalities have been described in patients in whom CGD is caused by loss of $p22^{phox}$, and therefore loss of NOX1, NOX2, NOX3, and NOX4 function, suggesting that room exists for safe inhibition of excessive ROS generation by NOX enzymes. Even if mice affected by mutations in $p22^{phox}$, NOXO1, or NOX3 genes show impaired otoconia formation and balance disorders, this side effect is unlikely to occur if a NOX inhibitor is taken at a later stage, because otoconia formation is a very early event in development (51, 72, 79).

Presently, no single available NOX-specific inhibitor is ready for use in clinical trials. The most widely used compounds in animal studies are DPI, which is an efficient NOX inhibitor, but nonspecific and toxic, and apocynin, which is relatively nontoxic, but for which inhibition of NOX enzymes has never been rigorously proven. Thus, professional and resourceful development of new, druglike NOX inhibitors through biotechnology start-ups and pharmaceutical companies will be extremely important. Novel compounds will have to show specificity and absence of toxicity and off-target effects, as well as *in vivo* efficacy in widely accepted animal models of disease. Therefore, a major challenge for the NOX scientific community will be the development of methods that

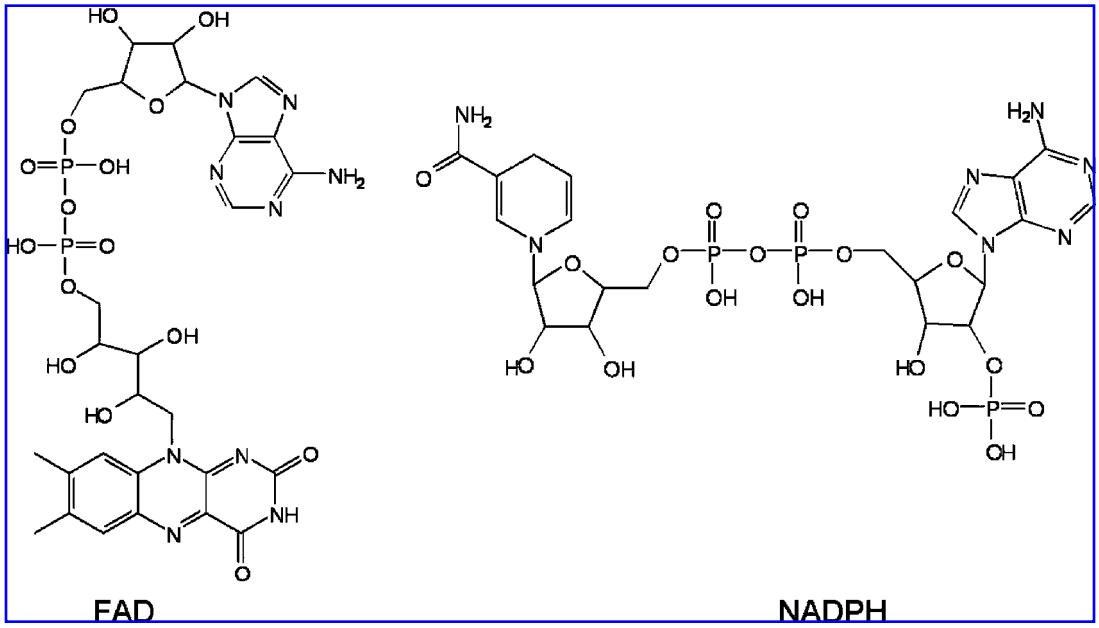


**FIG. 5. Two-dimensional structures of flavin adenine dinucleotide (FAD) and nicotinamide adenine dinucleotide phosphate (NADPH).**

allow detection of NOX activity *in vivo* in animals and in humans, going beyond presently available *ex vivo* technologies.

In summary, NOX inhibitors will not be in the clinics tomorrow. However, "*a change is gonna come*" (Sam Cooke, 1964, RCA Victor), and the day when clinicians will prescribe a NOX1 inhibitor for hypertension, a central nervous system–targeted NOX2 inhibitor for Alzheimer disease, or a NOX4 inhibitors for pulmonary fibrosis is on the horizon.

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**Abbreviations Used**

AEBSF = 4-(2-aminoethyl)-benzenesulphonyl fluoride
CGD = chronic granulomatous disease
DPI = diphenyleneiodonium
DUOX = dual oxidase
fMLF = formyl-methionyl-leucyl-phenylalanine
GTX = gliotoxin
HUVECs = human umbilical vein endothelial cells
INT = iodonitrotetrazolium
MPO = myeloperoxidase
NBT = nitroblue tetrazolium
NEM = *N*-ethylmaleimide
NOS = nitric oxide synthase
NOX = NADPH oxidase
PAO = phenylarsine oxide
PK = protein kinase
PL = phospholipase
PMA = phorbol myristate acetate
ROS = reactive oxygen species
SAH = subarachnoid hemorrhage
TBARS = thiobarbituric acid–reactive substances
TC = Tanimoto coefficient

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