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The Quest for Selective Nox Inhibitors and Therapeutics: Challenges, Triumphs and Pitfalls

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Abstract

Significance: Numerous studies in animal models and human subjects corroborate that elevated levels of reactive oxygen species (ROS) play a pivotal role in the progression of multiple diseases. As a major source of ROS in many organ systems, the NADPH oxidase (Nox) has become a prime target for therapeutic development. **Recent Advances:** In recent years, intense efforts have been dedicated to the development of pan- and isoform-specific Nox inhibitors as opposed to antioxidants that proved ineffective in clinical trials. Over the past decade, an array of compounds has been proposed in an attempt to fill this void. **Critical Issues:** Although many of these compounds have proven effective as Nox enzyme family inhibitors, isoform specificity has posed a formidable challenge to the scientific community. This review surveys the most prominent Nox inhibitors, and discusses potential isoform specificity, known mechanisms of action, and shortcomings. Some of these inhibitors hold substantial promise as targeted therapeutics. **Future Directions:** Increased insight into the mechanisms of action and regulation of this family of enzymes as well as atomic structures of key Nox subunits are expected to give way to a broader spectrum of more potent, efficacious, and specific molecules. These lead molecules will assuredly serve as a basis for drug development aimed at treating a wide array of diseases associated with increased Nox activity. *Antioxid. Redox Signal.* 20, 2741–2754.

Introduction

OXIDATIVE STRESS is manifested by a shift in the steady-state balance between the production of reactive oxygen or nitrogen species (ROS/RNS) and the antioxidant reserves of a biological system. When the proper cellular redox homeostasis is maintained, low levels of ROS evidently play an essential role as second messengers in myriad inter- and intracellular signaling cascades regulating neuronal signaling, blood pressure, and balance (52, 133). However, following a shift toward an increasingly pro-oxidant state, cells may succumb to an inexorable impairment of function and damage as a consequence of excessive protein and lipid oxidation, and DNA damage. Among the leading causes of death that afflict the U.S. population (72), cardiovascular diseases, neurodegenerative disorders, and cancer appear to share oxidative stress as a common nexus (18).

Excessive and unabated levels of ROS have been shown to play a key role in the pathophysiology of cardiovascular diseases, such as hypertension (6, 53, 99, 115, 160), atherosclerosis (149), cardiac hypertrophy (14), stroke (82), and conditions including ischemia reperfusion (110), and restenosis (76, 104). Moreover, the neurodegenerative Huntington's (164), Alzheimer's (9), and Parkinson's diseases have augmented ROS implicated in their etiology (27, 154). Further, evidence for the involvement of ROS in the progression of carcinogenesis is also demonstrated (172). Indeed, ROS can lead to oxidation of DNA resulting in gene mutations, duplication, and activation of oncogenes (87).

However, physiological ROS levels are demonstrated to regulate signaling pathways (52, 133) *via* thiol modification of redox-sensitive proteins, resulting in conformational changes that alter enzymatic activity (kinases and phosphatases involved in growth factor signaling) or DNA binding of activated transcription factors, such as NF κ B and AP-1 (129, 176).

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The main cellular defense mechanisms that protect against increased ROS levels are antioxidant enzymes, such as superoxide dismutase, catalase, glutathione peroxidases, and thioredoxin as well as dietary scavengers, including α -tocopherol and ascorbic acid (52). Importantly, their roles in the neutralization of ROS derived from cellular respiration and other enzymatic sources, such as xanthine oxidase, uncoupled NO synthase, and, most important to this review, NADPH oxidase (Nox) are well established (47). Under normal homeostatic conditions, organ systems utilize these antioxidant systems to maintain the redox balance. Increasing evidence demonstrates Nox as a main cellular source of ROS, playing an important role in ROS-dependent signaling cascades (20, 98). Moreover, it is becoming increasingly evident that augmented ROS production by the Nox family of proteins promotes activation and upregulation of Nox isoforms in a “feed-forward” mechanism further contributing to oxidative stress and disease progression (40, 98).

Nox Family of Proteins

Nox enzymes belong to a closely related family of membrane proteins that catalyze the production of superoxide anion and/or hydrogen peroxide by electron transfer from NADPH to molecular oxygen *via* heme groups in their transmembrane domains, utilizing FAD as a cofactor. To date,

seven members of the Nox family have been identified, namely, Nox1, Nox2, Nox3, Nox4, Nox5, Duox1, and Duox2. The isoforms differ in their subunit composition, activation, physiological and pathophysiological functions, and in their subcellular and tissue expression (25, 92) (Table 1). For more extensive details on the structure and activation of Nox isoforms, please refer to previous reviews (2, 13, 59, 66, 103, 152). The most well-studied Nox isozyme to date is the respiratory burst enzyme Nox2 (*aka* gp91^{phox}) originally identified in phagocytes (141) where it has been well characterized (33) and subsequently discovered in a wide range of other cell types (65, 114, 122, 123, 131). The catalytic core of Nox2 consists of two membrane-spanning subunits that together form the cytochrome b₅₅₈: one being the Nox2 subunit, a six transmembrane spanning protein containing NADPH- and FAD-binding sites on its C-terminal tail, and the other p22^{phox}, its stabilizing cohort. The activity of Nox2 is regulated by the cytosolic subunits p47^{phox} (organizer subunit), p67^{phox} (activator subunit), and p40^{phox}, and the small Rho-family GTP-binding protein Rac2 or Rac1. Beyond its major role in innate immune defense mechanisms, Nox2 is also involved in a wide array of well-regulated physiological processes in the cardiovascular system (139) and in the brain (148). Increased expression or deregulation of Nox2 in those tissues elicits profound health implications (18). The Nox1 system is comprised of membrane subunits Nox1 and p22^{phox} and in its canonical

TABLE 1. NOX FAMILY OF PROTEINS

Nox isozyme	Subunits/regulators	Tissue expression/ROS type	Main features
Nox1	p22 ^{phox} , NOXO1, NOXA1, Rac1 (canonical), or p22 ^{phox} , p47 ^{phox} , NOXA1, Rac1 (hybrid)	Heart, lungs, blood vessels, brain. Superoxide anion.	Depending on the cell type can utilize varied cytosolic subunits (canonical or hybrid). The canonical system is constitutively active. Involved in necrosis, hypertrophy, migration, and tissue growth.
Nox2	p22 ^{phox} , p47 ^{phox} , p67 ^{phox} , p40 ^{phox} , Rac1, or Rac2	Innate immune cells, heart, lungs, blood vessels, brain. Superoxide anion.	Prototype Nox. Tightly regulated. Involved in immune defense, angiogenesis, neurodegenerative disorders, and necrosis.
Nox3	p22 ^{phox} , NOXO1, NOXA1 (p47/p67), Rac1	Inner ear (cochlear and vestibular sensory epithelia). Superoxide anion.	Constitutively active. Involved in balance, otoconia biosynthesis, and potentially hearing loss.
Nox4	p22 ^{phox} , Poldip2	Heart, lungs, blood vessels, kidney, prostate. Hydrogen peroxide (?).	Involved in differentiation, migration, growth, and survival.
Nox5	No subunit identified to date. Regulated by Ca ²⁺	Cardiovascular system, spleen lymph nodes, testes. Superoxide anion.	EF hand domains. Involved in inflammatory gene expression, growth, and proliferation.
Duox1	DUOXA1 (for maturation)	Thyroid gland, airway epithelium, cerebellum, testis, prostate. Hydrogen peroxide.	EF hand domains and N-terminal peroxidase-like domain. Involved in thyroid function.
Duox2	DUOXA2 (for maturation)	Thyroid gland, airway epithelium, gastrointestinal epithelia, uterus, gallbladder. Hydrogen peroxide.	EF hand domains and N-terminal peroxidase-like domain. Involved in thyroid hormone synthesis.

(?)=noted controversy as to whether H₂O₂ or superoxide is the primary metabolite.
Nox, NADPH oxidase; ROS, reactive oxygen species.

conformation is regulated by cytosolic NOXO1 organizer (homologous to p47^{phox}), NOXA1 activator (homologous to p67^{phox}), and Rac1. However, increasing evidence implicates p47^{phox}-induced activation of the hybrid-Nox1 system in a variety of disease settings (35, 101, 120). As Nox1, Nox3 requires p22^{phox} and can be regulated by NOXA1 and NOXO1 but also by p47^{phox} and p67^{phox} (26, 163). Nox4, on the other hand, does require p22^{phox} but the only other known regulator described for it is Poldip2. Nox5, distinct from Nox1–4, does not require p22^{phox} and is regulated by calcium through EF-hand motifs present in its N-terminal region (11). Interestingly, Nox5 is the only isoform that is not expressed universally across mammalian species; that is, the Nox5 gene is absent in mouse and rat genome (12). Importantly, Nox5 is expressed in human vasculature and is abundant in lymphoid tissue and testes where it may be involved in spermatogenesis (11, 12, 25, 54, 142). Like Nox5, Duox1 and Duox2 do not require p22^{phox} for activity and they also possess EF-hands, which render them Ca²⁺-dependent enzymes (7). Distinguishing characteristics in Duox1 and 2 *versus* other members of the Nox family are that they contain seven membrane-spanning domains and a peroxidase-like domain in their extracellular N-terminal region. At this juncture, it is important to assert that an individual Nox isoform could have unique and opposing effects in a species-, cell-, and tissue-dependent manner. For a comprehensive review of the role each Nox isoform plays in cellular physiology and signaling cascades, the readers are directed to some recent reviews (93, 101) and other articles in this Forum.

Free-Radical “Scavengers” Versus Nox Inhibitors

Given the plethora of diseases involving oxidative stress and Nox, in particular, the need for delicate maintenance of cellular redox balance and prevention of harmful overproduction of ROS has become increasingly clear (165). Initial attempts to achieve this goal and prevent or reverse the damaging effects of ROS involved the use of antioxidants, such as vitamins E, C, and A, which *in vitro* and in animal studies proved beneficial (8, 162). Moreover, many observational studies over the years have propounded the benefits of an antioxidant-rich, for example, Mediterranean diet (83). However, data from randomized clinical trials using these vitamins reported no effects in improving cardiovascular disease outcomes and in some cases have paradoxically indicated deleterious effects. Two of the most notorious of these include reports of elevated heart failure rates in patients who took high dose of vitamin E (108) and an increased incidence of cancer in smokers supplemented with beta-carotene (68). The reason for the failure of these clinical trials is not entirely clear, but a general consensus in the field points to multiple flaws in study design (28). Moreover, scavenging a particular ROS moiety without regard for the potential consequence of generating another reactive species metabolite is seriously misguided (36, 159), and perhaps the most obvious of these flaws is our contention of an inadvertent disruption of salutary ROS signaling in clinical studies (28). In that regard, the popular mantra of “more is better” should be treated with healthy skepticism when it comes to both antioxidants and Nox inhibitors. Further, a growing body of evidence demonstrates a beneficial role for exogenously supplied catalase or the ROS scavenger Tempol in inhibiting the oxidative burden associated with “feed-forward” Nox activation and expres-

sion both *in vitro* and *in vivo* (42, 111, 112). This may include redox-sensitive pathways upstream or linking one Nox to another. For example, ROS are known to activate key kinases, that is, c-Src (61), and/or elicit the release of calcium (180) that is essential for Nox5 and Duox activities. However, the prudent use and extent of global ROS scavenger administration remains important as many ROS-generating enzymes contribute to salutary redox-signaling pathways under normal physiological conditions. Thus, it would appear that the preferred strategy to combat the deleterious consequences of oxidative stress is to directly assess the source of ROS generation, which in most of the systems involves one or more of the Nox isozymes (48). In fact, a quest for specific Nox inhibitors has been underway for more than two decades (31) and presently, it has intensified across scientific disciplines. Indeed, targeting Nox activity has great therapeutic potential for numerous disorders, including pancreatic cancer (166), hypertension (28, 124), cystic fibrosis (130), Parkinson's disease (27), acute lung injury (22), pulmonary fibrosis (71), heart failure (179), ischemia/reperfusion injury (88), chronic kidney disease (62), and stroke-related neurodegeneration (132) to name just a few. Since the regulation and structure of Nox isozymes is complex, there are many possible strategies to achieve inhibition (138). For example, one could target upstream regulators such as receptor modulators of Nox activity, like the angiotensin receptor (24, 158) and calcium channel blockers (51, 75), or inhibit kinases involved in the recruitment of cytosolic subunits to their respective Nox subunit (84, 102). Further, modification of mitochondrial-Nox crosstalk is another suggested mode of Nox activity modulation (91, 94). However, more studies are required to fully elucidate the dynamic nature surrounding this process, as well as the Nox isoforms involved. Also, inhibiting transcription processes that increase Nox expression or increase degradation of Nox subunits would lead to attenuated ROS production. All such tactics are expected to be problematic as they are involved in many other signaling cascades independent of Nox and their inhibition is likely to cause untoward effects. Similarly, compounds that antagonize key regulatory subunits, such as p47^{phox}, unless specifically targeting their interaction with other key Nox components (*e.g.*, Nox2) may have undesirable effects as it has become evident that individual Nox subunits may have Nox-independent functions (126). Thus, the need to directly target Nox-specific ROS-generating activity comes into greater focus.

Development of Nox Inhibitors (Peptidic Versus Small-Molecule Inhibitors)

An ideal Nox inhibitor would not inhibit other sources of ROS such as xanthine oxidase, would be devoid of ROS-scavenging or cytotoxic properties, would efficaciously and specifically target a unique Nox isoform, would be druggable, and would possess ideal pharmacokinetic characteristics. Despite the intense efforts dedicated to the identification of such an inhibitor, the task has proven extremely difficult. To date several molecules have been identified as Nox inhibitors; however, most of these are not specific. Nevertheless, a few hold significant promise. Many reviews have been dedicated to describing the available Nox inhibitors in recent years (29, 48, 78, 85, 89) and since then relatively few new compounds have come to the forefront. The following discussion is an effort to be as comprehensive and up-to-date on these developments.

Peptidic inhibitors

Attempts to develop Nox-specific peptidic inhibitors have been made since 1990 (136, 143). Peptide inhibitors of Nox have been extensively reviewed elsewhere (29, 37, 50). Interestingly, many examples of peptidic inhibitors that span regions of the Nox subunits themselves have been reported by the Quinn (41) and Pick (38, 39, 117) laboratories using phage display and peptide-walking techniques. Among these, the first peptide to be developed as a cell-permeant Nox-Specific inhibitor and potential therapeutic is Nox2ds-*tat* ([H]-R-K-K-R-R-Q-R-R-R-C-S-T-R-I-R-R-Q-L-[NH₂], previously gp91ds-*tat*) (134).

Nox2ds-*tat*. More than 10 years after its development, Nox2ds-*tat* remains a widely utilized isoform-specific inhibitor of Nox2-derived ROS. Nox2ds-*tat* is a chimeric peptide that contains a nine-amino-acid sequence recapitulated from the cytosolic loop B of Nox2 (amino acids 86–94, underlined) and a nine-amino-acid subdomain of HIV-*tat* transport region that confers the peptide the ability to be internalized by cells (134, 135). Nox2ds-*tat* specifically blocks the interaction between Nox2 and p47^{phox} and selectively blocks Nox2 activity with an IC₅₀ of 0.7 μ M (35). Nox2ds-*tat* does not inhibit Nox1- or Nox4-derived ROS generation; neither has it any effect on xanthine oxidase activity (35). Perhaps the best evidence of its specificity is that this inhibitor does not inhibit the canonical Nox1 oxidase that utilizes NOXO1 or the hybrid Nox1 oxidase that utilizes p47^{phox} as its organizing subunit. The inhibitory effects of Nox2ds-*tat* have widely been demonstrated both *in vitro* as well as *in vivo*. *In vitro* assays indicate that Nox2ds-*tat* inhibits superoxide anion production in endothelial cells in response to various stimuli, including nutrient deprivation (109), hypoxia (4), atrial natriuretic peptide (55), angiopoietin-1 (70), interleukin-4 (170), shear stress (49), and calcineurin inhibitors (95). Nox2ds-*tat* also blocks angiotensin II-induced superoxide production in human resistance artery smooth muscle cells (161) and collagen-induced Nox activity in platelets (96). *In vivo*, subcutaneous infusion of Nox2ds-*tat* attenuated superoxide production in a variety of disease models (45, 76, 81, 105, 106, 173). More recently, Nox2ds-*tat* has been used to demonstrate the role of Nox2 in insulin-resistance-related endothelial cell dysfunction (151), triggering of inflammasome activation (1), and to study the interplay between platelets, TNF α , and Nox2 in heart failure (21).

Theoretically, by their nature, peptides may be rationally designed to better target protein–protein interactions within the enzyme complex important for Nox activity and thus, uniquely block only those sites that are involved in the assembly of the active complex. In practice, however, this may not be as straight forward. For example, a similar strategy that we applied to the design of Nox2ds-*tat* did not yield peptide inhibitors for Nox4. As we explain in that study, this could possibly be explained by a tightly assembled and active conformation of Nox4 that, unlike other Noxes, cannot be disrupted by conventional means (34). As potential drug therapies, peptidic inhibitors have historically been disregarded for their perceived intrinsic disadvantages; one of the most important of which is their suspected inability to penetrate the plasma membrane, a property dependent on sequence, charge distribution, and hydrophobic properties. Peptidic inhibitors are also judged at a disadvantage due to their poor oral bioavailability since peptides are readily de-

graded in the digestive system. However, great strides have been made in recent years to develop stabilization methodologies and alternative delivery systems that are expected to obviate these limitations (116, 169). Moreover, studies in our laboratory have indicated promising preliminary outcomes of right heart and left heart function preservation in two distinct models of pulmonary hypertension by aerosolization of Nox2ds-*tat* in mice (unpublished observations). It remains to be seen, therefore, whether these exciting and promising results hold true under scrutiny.

Small-molecule inhibitors

Diphenylene iodonium and apocynin. The traditionally used inhibitors of Nox activity are diphenylene iodonium (DPI) and apocynin (Fig. 1). Although widely utilized to investigate the role of the Nox family of proteins in a given pathway, their many limitations are increasingly becoming evident as their mechanisms of action are being appreciated (3).

DPI was first identified as a potent Nox inhibitor in a cell-free preparation of neutrophil membranes (32, 46) and was shown to form adducts with FAD to inhibit ROS formation (121). However, despite the fact that DPI is also an irreversible and nonselective inhibitor of flavin-dependent enzymes, including nitric oxide synthase and xanthine oxidase, its inhibitory effects continue to be inappropriately interpreted as evidence of Nox activity. These off-target effects nullify its potential as a therapeutic candidate. That said, in a recent study, DPI and its analog di-2-thienyliodonium (DTI) were shown, in the nanomolar range, to exhibit antineoplastic properties, decreasing colon cancer cell proliferation by blocking cell cycle progression at the G1/S interface and not just to decrease ROS levels but also Nox1 expression (44). Perhaps the emphasis here should be placed on the use of very low concentrations of the iodoniums that may prove relatively selective for Nox *versus* other flavoproteins. Nevertheless, it is difficult to fathom the systemic administration of these agents *in vivo* where many other enzymes of importance could be inhibited.

Perhaps the most controversial inhibitor of Nox activity to date is apocynin. Unlike DPI, many of apocynin's users have assumed that its effects are specific to Nox based on early reports in cell-free systems in which it was shown to inhibit the translocation of p47^{phox} to plasma membranes (thereby inhibiting Nox2 activation). Apocynin is a natural methoxy-substituted catechol isolated from *Picrorhiza kurroa* that was shown to inhibit superoxide anion production *in vitro* and to

FIG. 1. Traditional inhibitors of Nox. Nox, NADPH oxidase.

have anti-inflammatory activity *in vivo* (155). This inhibitory effect required, however, activation by myeloperoxidase (146). Thus, apocynin has little or no effect in cells that do not express this peroxidase activity, which likely describes its widely variable activity across cells, tissues, and species. Further, apocynin can act as an antioxidant, *per se* (73), and in fact, it has notable capacity ($>100\ \mu\text{M}$) as a scavenger of nonradical oxidant species, such as HOCl and H_2O_2 (128). Many of the nonspecific effects of apocynin have been well documented elsewhere (3), so it is somewhat surprising that the compound continues to be widely used to dissect the role of Nox both *in vitro* as well as *in vivo* studies. Without question, the results obtained utilizing apocynin require careful interpretation when implicating a role of Nox, *per se*, in experimental studies. At minimum, its effects should be validated with a more specific Nox2 inhibitor or using cells genetically void of Nox. On a positive note, however, the knowledge that as a result of peroxidase metabolism, apocynin yields reactive quinones that bind cysteine residues in p47^{phox} and block its translocation to the membrane has generated a model of interaction between apocynin analogs and p47^{phox} using computational analysis (79). This model and computational screening could lead to the identification of new analogs that inhibit the hybrid Nox1 and canonical Nox2 (isoforms activated by p47^{phox}) and do not exhibit the undesirable properties described for apocynin. Until that time, apocynin should not be classified as a Nox-specific inhibitor (73). In summary, this discussion is not intended to dismiss the many important studies that employ apocynin and implicate Nox involvement in a variety of cell-signaling pathways and pathologies. However, with the advent of more specific Nox isoform inhibitors, the use of apocynin should likely be avoided.

S17834 and AEBSF. Another compound potentially displaying a broad profile of effects is S17834 (23), a polyphenol inhibitor of superoxide formation by Nox with an $\text{IC}_{50} > 25\ \mu\text{M}$, which also increases SIRT1 deacetylase activity, LKB1 phosphorylation at Ser428, and AMPK activity (74, 177, 178). Yet, despite its protective role in a range of vascular disorders, its precise mechanism of Nox modulation remains unknown. Additionally, AEBSF inhibits binding of cytochrome b_{558} to p47^{phox} , but it is a known serine protease inhibitor (43). Resultantly, its use as a tool to delineate the precise role of Nox in disease models is limited due to its multiple effects in cells (175).

As the demand for Nox inhibitors has increased dramatically in recent years, major efforts have been dedicated to high-throughput screening (HTS) and development of orally bioavailable lead molecules both in academia and the pharmaceutical industry. From these laborious endeavors, a few compounds have emerged with therapeutic potential.

Triazolo pyrimidine derivatives: VAS2870 and VAS3947. Two triazolo pyrimidine derivatives VAS2870 and VAS3947 have been identified by Vasopharm as Nox inhibitors and at least VAS2870 has been made commercially available (Fig. 2). VAS2870 [3-benzyl-7-(2-benzoxazolyl)thio-1,2,3-triazolo[4,5-d]pyrimidine] was first characterized as a Nox inhibitor, without antioxidant properties or attenuation of xanthine oxidase activity, and was shown to effectively suppress growth factor-mediated ROS generation and vascular smooth muscle cell

FIG. 2. Triazolo pyrimidines.

migration (156). In a neutrophil-cell-free system, VAS2870 inhibited superoxide production with an IC_{50} of $10.6\ \mu\text{M}$. These results appear inconsistent with findings from Gatto *et al.* who reported lack of inhibition of Nox2 by VAS2870 in a semipurified enzyme preparation (58). This difference may be a consequence of the dynamics of complex assembly that is dependent on the order of addition of the stimulus. VAS2870 ($50\ \mu\text{M}$) also inhibits the stimulation of vasculogenesis by PDGF-BB of mouse embryonic stem cells (100), and it inhibits wound-margin H_2O_2 production without obvious toxicity in zebrafish larvae (119). At $10\ \mu\text{M}$ it inhibits Nox activity in ox-LDL-exposed human umbilical vein endothelial cells (150). VAS2870 was shown to inhibit Nox4-derived ROS production in the sarcoplasmic reticulum of mammalian skeletal muscle and it abolished O_2 -coupled redox regulation of the ryanodine receptor- Ca^{2+} channel (RyR1). It was also found that VAS2870 directly caused thiol alkylation modification of RyR1 (153). Thus, the off-target effects of VAS2870, such as the direct effect on the cellular thiol redox status, must be taken into consideration when assessing the agent's utility. Nevertheless, VAS2870 should be considered a *pan*-Nox inhibitor, which in its own right may prove very useful in certain disease states. Indeed, it was shown to inhibit Nox1-, preassembled Nox2-, as well as Nox4- and Nox5-dependent ROS production (5). Similarly, VAS3947, a VAS2870 analog with improved solubility, is also considered a *pan*-Nox inhibitor as it inhibited ROS generation in three cellular models with varied expression patterns of all known Nox isoforms (175).

Pyrazolopyridine derivatives: GK-136901 and GKT137831. HTS of small-molecule libraries and subsequent optimization of lead compounds has identified dual Nox1/4 inhibitors (56, 97). Among these are GKT136901 [2-(2-chlorophenyl)-4-methyl-5-(pyridin-2-ylmethyl)-1H-pyrazolo [4,3-c]pyridine-3,5(2H,5H)-dione] and GKT137831, which now represent the first orally-active dual Nox1 and 4 inhibitors (Fig. 3) (10, 63, 80). GKT137831 is a candidate drug currently being developed as a new therapy for diabetic nephropathy and reportedly undergoing phase I clinical testing (174). As the first pyrazolopyridine derivative described, GKT136901 was found to be a Nox inhibitor with high degree of potency for Nox4 (inhibitory constant $[K_i] = 165 \pm 5\ \text{nM}$) and Nox1 ($K_i = 160 \pm 10\ \text{nM}$) and with a 10-fold selectivity over Nox2 ($K_i = 1530 \pm 90\ \text{nM}$) while exhibiting no inhibitory effects on other ROS-producing enzymes, redox-sensitive enzymes, or other proteins (140). CD44-hyaluronic acid-dependent gene regulation role in atherosclerosis *via* Nox1/4 activation was first demonstrated by the attenuation of ROS generation and atherosclerosis by GKT136901 (168). Moreover,

FIG. 3. Pyrazolopyridine derivatives.

administration of GKT136901 reduced angiogenesis and tumor growth *in vivo* in a PPAR α -dependent manner, confirming the role of Nox1 in endothelial cell migration and angiogenesis (57). GKT136901 has also been shown to inhibit Nox5 ($K_i = \sim 450$ nM) in a recent study by Musset *et al.* (118). The study used GKT136901 to investigate whether Nox5 accounts for Ca^{2+} -dependent superoxide production in spermatozoa, where Nox1 or Nox4 is not detectable. GKT136901 completely inhibited sperm cell motility induced by hydrogen peroxide and other important cell functions mediated by Nox5 (118).

The second pyrazolopyridine derivative GKT137831 (Fig. 3) has been used to demonstrate the role of Nox in liver fibrosis and hepatocyte apoptosis (10, 80). Similar to GKT136901, GKT137831 was shown to be a highly potent inhibitor of human Nox4 ($K_i = 140 \pm 40$ nM) and human Nox1 ($K_i = 110 \pm 30$ nM) and was found to be at least 10-fold less potent on Nox2 ($K_i = 1750 \pm 700$ nM) and 3-fold less potent on Nox5 ($K_i = 410 \pm 100$ nM) (10). GKT137831 was also used to study the role of Nox4 in hypoxia-induced pulmonary vascular cell proliferation (64) where it attenuated hypoxia-induced H_2O_2 production, proliferation, and TGF- β 1 expression. In the same study, it was shown *in vitro* to blunt reductions in PPAR γ in HPAECs and HPASMCs, while *in vivo*, it inhibited hypoxia-induced increases in TGF- β 1, reduced PPAR γ expression, and attenuated right ventricular hypertrophy and pulmonary artery wall thickness (64). GKT137831 has also been used to investigate the role of Nox1 in diabetes-accelerated atherosclerosis as it prevented oxidative stress in response to hyperglycemia in human aortic endothelial cells (63). However, while these models of Nox-associated disease show a protective role of GKT136901 and GKT137831 by modulating ROS levels, their precise mode of inhibition remains to be demonstrated. A new report that addresses the pharmacology of this compound provides evidence of its property as a selective scavenger of peroxynitrite (137).

ML171. To our knowledge, only one small-molecule isoform-specific Nox inhibitor has been reported so far, namely, ML171 (2-acetylphenothiazine) (Fig. 4). This compound belongs to a subset of phenothiazines and is identified as a selective Nox1 inhibitor. ML171 has an IC_{50} for Nox1 in the submicromolar range, that is, $0.129 \mu\text{M}$ in HT29 cells and $0.25 \mu\text{M}$ in a HEK293-Nox1 reconstituted cell system, ~ 20 -fold higher for Nox-2, -3, and -4 as well as for xanthine oxidase (60). ML171's mechanism of action may involve interaction with the Nox1 catalytic subunit since only overexpression of this subunit overcomes the ML171-dependent

FIG. 4. Phenothiazine derivative.

inhibition of ROS generation in heterologous Nox1-expressing HEK293 cells (60). Further, ML171 blocked Nox1-dependent extracellular matrix-degrading, actin-rich cellular structures (invadopodia) in colon cancer cells, providing support for the potential therapeutic use of this drug (60). Despite the commercial availability of this compound, only two studies to our knowledge have been published using ML171 (112). In that study, ML171 was utilized to investigate the reciprocal relationship between mitochondrial- and/or Nox-derived ROS and cyclooxygenase-2 in vascular dysfunction and hypertension. Another report utilized ML171 to inhibit collagen- and fibrinogen-dependent ROS production in platelets that express both Nox1 and Nox2 (167). In this study, $0.5 \mu\text{M}$ of ML171 totally abolished intracellular ROS production in response to platelet adhesion to either collagen or fibrinogen.

Fulvene-5. Triphenylmethane dyes, such as brilliant green and gentian violet that have chemical similarity to DPI were shown to be potent and efficacious inhibitors of Nox2 and Nox4 activity (127). Development of fulvene derivatives by a structure-based approach generated a new class of inhibitors. One of these derivatives is fulvene-5 (Fig. 5) that at $5 \mu\text{M}$ equally inhibited Nox2 and Nox4 *in vitro*, and when applied *in vivo*, successfully blocked the growth of endothelial tumors of mice (15). Reportedly, this was most likely through its ability to inhibit Nox4, since the effect of fulvene treatment was consistent with the results obtained using Nox4 shRNA (15). Since then, no further reports have been published; information about the specificity of fulvene-5, mechanism of

FIG. 5. Other Nox-inhibiting compounds.

action, and full pharmacological profile for potency, efficacy, and cytotoxicity are lacking.

Naloxone. Naloxone (Fig. 5), a commonly used antagonist of opioid receptors, was found to be highly effective in preventing dopaminergic degeneration in different models of rodent Parkinson's disease by inhibiting inflammatory responses (107). In search for the potential neuroprotective action of naloxone, it was found that it binds to Nox2 and blocks translocation of $p47^{phox}$ to the plasma membrane, leading to inhibition of ROS production (171). In fact, the neuroprotective effect of this drug is dependent on Nox2 but independent of opioid receptors since (+)-naloxone, the inactive isomer for the activation of opioid receptors, is as potent as (–)-naloxone at inhibiting ROS production and binding to Nox2. In fact, the IC_{50} for both isomers was closed to $2\text{ }\mu\text{M}$. It is interesting to note that naloxone was also effective at inhibiting the activity of preassembled enzyme (171). That said, no reports could be found that compare its binding or inhibition of other Nox subunits.

Celastrol. A triterpenoid antioxidant compound celastrol (Fig. 5), isolated from the Chinese Thunder of God vine (*Tripterygium wilfordii*), has been used in traditional Chinese medicine for its beneficial and curative effects of various inflammatory diseases and cancer (19). Recently, it has been shown that celastrol is, in fact, a potent Nox inhibitor in general but with preference against Nox1 and Nox2 (Nox1 $IC_{50}=0.41\pm0.20\text{ }\mu\text{M}$, Nox2 $IC_{50}=0.59\pm0.34\text{ }\mu\text{M}$, Nox4 $IC_{50}=2.79\pm0.79\text{ }\mu\text{M}$, and Nox5 $IC_{50}=3.13\pm0.85\text{ }\mu\text{M}$) (77). Analysis of enzyme kinetics showed positive cooperativity in its inhibition of Nox1 and 2 activity (77). Paradoxically, celastrol was found to suppress the viability of breast cancer MCF-7 cells while stimulating ROS production (86). Further, Hansen *et al.* demonstrated that celastrol is able to act on several distinct stress response pathways and identified numerous cellular effects of celastrol (69), thus, questioning the value of this compound as a Nox-specific inhibitor. Nonetheless, it is possible that structural activity relationship (SAR) studies may identify analogs of celastrol that selectively inhibit Nox.

Ebselen. Ebselen is yet another compound that has been described as a Nox inhibitor but has previously been characterized as having unrelated effects (Fig. 5). Identified as a glutathione peroxidase mimetic (145), ebselen and its selenium- (but not sulfur-) containing analogs are able to consume hydrogen peroxide in a catalytic cycle that utilizes thiol-containing compounds, such as glutathione, as a substrate (125, 145). Recently however, using an *in vitro* fluorescence polarization Nox2 assay, Smith *et al.* identified ebselen and some of its analogs as potent Nox2 inhibitors that, depending on the congener, inhibited Nox1, 4, and 5 activity at substantially lower potency (147). One of these compounds, JM-77b, displays an especially attractive profile of selectivity for Nox2 ($IC_{50}=0.4\text{ }\mu\text{M}$) compared with Nox1 ($IC_{50}=6.3\text{ }\mu\text{M}$), Nox5 ($IC_{50}=17\text{ }\mu\text{M}$), and Nox4 (no significant inhibition) (147). Smith *et al.* also showed that ebselen blocked the translocation of $p47^{phox}$ to neutrophil membranes, thus, interfering with the assembly of Nox2 and suggesting that ebselen and its congeners could represent a class of compounds with significant therapeutic potential (147). However, further studies are required to demonstrate the mechanism of action

of ebselen, or its analogs, on $p47^{phox}$ activation due to the recent demonstration that $p47^{phox}$ may still translocate to the plasma membrane independently of the indicated ebselen site of inhibition (157). Moreover, until which time a congener can be found that is devoid of peroxidase mimetic activity, some caution should be applied when interpreting its effects.

Tetrahydroquinolines. Recently, work published by our group demonstrated the effect of bridged tetrahydroquinolines as selective Nox2 inhibitors (30). In this report, ($\pm$)-(1*S*,4*R*,9*S*)-5-bromo-3,3-dimethyl-9-(2-methylallyl)-10-pentyl-1,2,3,4-tetrahydro-1,4-(epiminomethano)naphthalene (compound 11g) and ($\pm$)-(1*S*,4*R*,9*S*)-5-bromo-3,3-dimethyl-9-(2-methylallyl)-10-(thiophen-2-ylmethyl)-1,2,3,4-tetrahydro-1,4-(epiminomethano)naphthalene (compound 11h) (Fig. 6) exhibited selective inhibition of Nox2 in intact Cos-Nox2 cells stimulated with phorbol-12-myristate-13-acetate ($IC_{50}\text{ }20\pm1.9$ and $32\pm1.9\text{ }\mu\text{M}$, respectively). Importantly, these compounds were unable to inhibit ROS production from Nox1-, Nox4-, and Nox5-expressing cells and displayed no free-radical scavenging effect or cytotoxicity in preliminary screens. These early data suggest that bridged tetrahydroquinolines represent a new group of compounds with potential to serve as a platform for developing therapeutic agents for the treatment of Nox2-dependent oxidative stress disorders. Thus, full assessment of specificity, *in vivo* efficacy, and pharmacokinetic properties are warranted.

Other Nox inhibitors. In a recent study, Borbely *et al.* (16) showed the methodical development of derivatives of primary hits obtained by an HTS campaign that searched for small-molecule inhibitors of Nox4. The most potent compounds belong to the following core structures: oxalyl hydrazides, flavonoids, oxindoles, benzoquinolines, and benzothiophenes. The best hit molecules identified by SAR analysis shared a 3D structure consisting of four pharmacophore points that feature hydrogen bond donors, acceptors, and two aromatic rings independent of the core structure. Although these studies shed light on the structural requirements for inhibitors under the conditions tested, there is no information to our knowledge on the specificity of these compounds for Nox4 as compared with other Noxes or other ROS-generating enzymes.

One of the major complications of HTS of small-molecule libraries is that once a hit is identified, it is necessary to rule out cell/organ toxicity before it can be deemed useful as a drug. In an attempt to avert this problem, screening a subset of natural compounds derived from edible plants that *per se* may not be toxic has been an alternative strategy. This strategy has been used to characterize inhibitors of Nox4 (90); as such, several diarylheptanoids and lignans were identified with relative specificity toward Nox4 and with potency

FIG. 6. Tetrahydroquinolines.

comparable to that of GKT137831. One caveat, however, is that one compound isolated from or mimicking a naturally occurring moiety from an edible source may not, on its own, be nontoxic. More broadly speaking, simple designation as “naturally occurring” does not necessarily deem a compound safe. If that were the case, venoms and plant-derived toxins would be considered intrinsically safe for humans. Therefore, while the concept of such exploration from natural products is rational, exciting, and potentially fruitful, it does not come without risk.

The last compound to be discussed here, although yet to be referred to as a Nox inhibitor, is 3-amino-3-(4-fluoro-phenyl)-1*H*-quinoline-2,4-dione; a novel synthetic compound that inhibits cisplatin-induced hearing loss by suppression of ROS, implicating perhaps inhibition of Nox3 function (144). Further analysis of this compound in terms of its direct effect on Nox3 *versus* other Nox isoforms is necessary.

Future Considerations for Specific Nox Inhibitors

Current Nox inhibitor strategies look to characterize inhibition as a read-out of modulated Nox-derived ROS in a variety of cell types and cellular systems that overexpress a single Nox isoform, followed by cytotoxicity analysis (56, 60, 156). However, little mechanistic data are demonstrated for their mode of inhibition and therefore their characterization remains incomplete. To date, many Nox inhibitors display a lack of specificity for a single Nox isoform (48). Interestingly, of the developed Nox inhibitors, many compounds share structural similarities to nucleotides; therefore, from the interaction with Nox-nucleotide domains, we may potentially infer their inhibitory mechanisms and explain the lack of isoform specificity. However, this remains to be supported through binding analysis that requires improved investigatory tools. Importantly, work by Smith *et al.* details the first technological advance for the development of p47^{phox}-dependent Nox inhibitors independent of ROS analysis alone (147), and provides a reliable assay to infer potential modes of inhibition. As our understanding of the Nox family of enzymes improves, we anticipate the development of similar tools delineating chemical interaction, which, when used in conjunction with “activity” assays, will overcome the limitations of current screening programs that solely investigate ROS detection.

With the technological developments and growing knowledge of structure and key interactions between subunits and regulators, a new approach to target these interaction mechanisms is gaining traction. This is the rational design and use of computational capabilities for virtual screenings of thousands of compounds; the only limiting factor of this strategy is the availability of NMR or X-ray crystal-based structures for the targeted proteins or protein–protein interactions. An exciting example of such a strategy was reported by Bosco *et al.* on an *in silico* screen to identify inhibitors of the Rac1–p67^{phox} interaction (17). The analysis was based on the X-ray crystal structure of the Rac1 and p67^{phox} complex, and using docking simulations the authors were able to virtually screen over 300,000 compounds. Subsequent biochemical assays and SAR analysis of initial hits allowed the identification of Phox-I1 class of inhibitors of Nox2. Virtual screening to identify small molecules that target the catalytic core of the

enzyme, although elegant and highly efficient, is not feasible at this time as membrane-spanning subunits of Nox have not been crystallized. However, many core protein interactions between the cytosolic Nox subunits have been elucidated (66, 67, 113); therefore, we anticipate increased interest in specific inhibitors of Nox subunit interactions in the near future using a similar technology. Importantly, this could be one strategy to develop isoform-specific inhibitors as many Nox systems have distinct activation mechanisms.

Conclusions

Despite the great efforts by many laboratories dedicated to the development of isoform-specific inhibitors of Nox, the task has proven very challenging as only a handful of lead compounds described actually target Nox activity specifically. Much work is still necessary to delineate the mechanism of action of the most promising inhibitors and to optimize their pharmacological and pharmacodynamics properties for their use as viable therapeutic tools for the treatment of oxidative-stress-associated disorders. That notwithstanding, all of the compounds discussed here have contributed to a better understanding of the regulation and function of the Nox family of proteins, both in their roles in signal transduction as well as disease. From that perspective, we remain hopeful that Nox-based therapies will be available in the near future and will have a profound impact in alleviating human disease.

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Abbreviations Used

DPI = diphenylene iodonium
DTI = di-2-thienyliodonium
HTS = high-throughput screening
K_i = inhibitory constant
Nox = NADPH oxidase
RNS = reactive nitrogen species
ROS = reactive oxygen species
RyR1 = ryanodine receptor-Ca²⁺ channel
SAR = structural activity relationship