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Inhibiting the Activity of NADPH Oxidase in Cancer

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Abstract

Significance: The primary function of NADPH oxidases (NOX1–5 and dual oxidases DUOX1/2) is to produce reactive oxygen species (ROS). If inadequately regulated, NOX-associated ROS can promote oxidative stress, aberrant signaling, and genomic instability. Correspondingly, NOX isoforms are known to be overexpressed in multiple malignancies, thus constituting potential therapeutic targets in cancer.
Recent Advances: Multiple genetic studies aimed at suppressing the expression of NOX proteins in cellular and animal models of cancer have provided support for the notion that NOXs play a pro-tumorigenic role. Further, large drug screens and rational design efforts have yielded inhibitor compounds, such as the diphenylene iodonium (DPI) analog series developed by our group, with increased selectivity and potency over ''first generation'' NOX inhibitors such as apocynin and DPI.
Critical Issues: The precise role of NOX enzymes in tumor biology remains poorly defined. The tumorigenic properties of NOXs vary with cancer type, and precise tools, such as selective inhibitors, are needed to deconvolute NOX contribution to cancer development. Most NOX inhibitors developed to date are unspecific, and/or their mechanistic and pharmacological characteristics are not well defined. A lack of high-resolution crystal structures for NOX functional domains has hindered the development of potent and selective inhibitors.
Future Directions: In-depth studies of NOX interactions with the tumor microenvironment (*e.g.*, cytokines, cell-surface antigens) will help identify new approaches for NOX inhibition in cancer. *Antioxid. Redox Signal.* 33, 435–454.

Keywords: NADPH oxidase, dual oxidase, cancer, inflammation, reactive oxygen species, small-molecule inhibitors

Introduction

CELLULAR REACTIVE OXYGEN SPECIES (ROS) such as hydrogen peroxide (H_2O_2) and superoxide anion ($O_2^{\bullet-}$) are generally produced in response to cytokine or growth factor stimuli, or, in the case of $O_2^{\bullet-}$, as a by-product of mitochondrial oxidative phosphorylation (144). As such, ROS play the role of secondary messengers involved in the regulation of cell differentiation, growth, migration, and host defense (29, 66, 111). Under physiological conditions, ROS levels are tightly regulated through the action of various scavenging and antioxidant systems to mediate the normal activity of cellular signaling pathways (144). Enzymatic antioxidants such as glutathione peroxidases, catalase, and superoxide dismutase; and non-enzymatic antioxidants such as reduced glutathione, thioredoxins, peroxiredoxins, and glutaredoxins ensure that the redox balance of the cell is maintained (174).

Dysregulation of redox homeostasis due to increased ROS production or decreased cellular antioxidant capacity leads to pathological oxidative stress and oxidation of cellular components, such as DNA, lipids, and proteins (21). An aberrant oxidant environment has been linked to the development of a wide array of disease states, including hypertension, diabetes, neurogenerative disorders, chronic inflammation, and cancer (4, 71, 80, 130). Correspondingly, elevated ROS levels have been detected in cancer cells, resulting from increased metabolism, enhanced mitochondrial activity, and mitogenic signaling (89, 165, 170).

Outside of mitochondria, NADPH oxidases (NOXs) are the primary source of H_2O_2 and $O_2^{\bullet-}$ in non-phagocytic mammalian cells (22, 105, 134, 147). Although they share

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some structural and functional domain elements, the seven members of the NOX family (NOX1–5, dual oxidases [DUOX] 1, and DUOX2) vary in their architecture, tissue distribution, and activation mechanisms. NOX enzymes generate $O_2^{\bullet-}$ by catalyzing the NADPH-dependent transfer of an electron to oxygen across the cell membrane using their shared ferric reductase-like transmembrane element, which contains four highly conserved heme-binding histidine residues, along with cytosolic flavin adenine dinucleotide (FAD)- and nicotinamide adenine dinucleotide (NAD)-binding domains (22, 147). NOX5 and the DUOX also contain Ca^{2+}-binding EF-hand motifs. Further, the highly homologous isoforms DUOX1 and DUOX2 have an additional extracellular peroxidase homology domain. DUOX1, DUOX2, and constitutively active NOX4 produce H_2O_2, either directly or *via* the spontaneous or enzymatic dismutation of $O_2^{\bullet-}$ (22, 60, 126, 127).

Study of the functional impact of NOX proteins and associated ROS on cancer signaling pathways has been hindered by the lack of specific, versatile, and high-quality monoclonal antibodies that are designed to recognize NOX proteins and confirm their expression in relevant model systems and tissues (4). Our research group has recently developed, validated, and made available for research use selective monoclonal antibodies targeting human DUOX1/2, NOX5, and NOX4 (8, 129, 189) (Fig. 1). The report of a validated human NOX1 monoclonal antibody also developed by our program will soon be published.

In addition to immunological means of detecting expressed NOX proteins, the ability to specifically and selectively inhibit NOX isoforms would allow for the unambiguous characterization of the role of NOX enzymes in cancer and could provide new therapeutic avenues for cancer treatment. At present, genetic depletion methods constitute a first-line, proof-of-concept method to help define the role of an NOX protein of interest in the etiology of malignancy. Such studies have revealed that the aberrant overexpression of NOX enzymes contributes to the etiology of many diseases (71, 127, 147, 190). Thus, NOX proteins are increasingly studied in the context of cancer. Effectively, the number of publications in PubMed referencing ''NADPH oxidase'' and ''cancer'' has been increasing exponentially since the discovery of $gp91^{phox}$ (NOX2) in 1986–1987 (55, 148, 171) (Fig. 2). In this work, we will review evidence supporting the NOX family as a therapeutic target in cancer, as well as past and ongoing efforts and shortcomings in inhibiting NOX activity and tumor growth using genetic and molecular approaches.

NOXs as Therapeutic Targets in Cancer

Role of NOX isoforms in cancer

Over the past 20 years, a large number of studies involving genetic manipulation of NOX expression coupled with modulation of activity using inhibitors have implicated NOX-mediated ROS (particularly from NOX1 and NOX4) in cell division, cell survival, angiogenesis, and integrin signaling, suggestive of a causal role in cancer development (42). The tissue and subcellular compartment distribution of NOX isoforms varies such that they affect cell signaling in

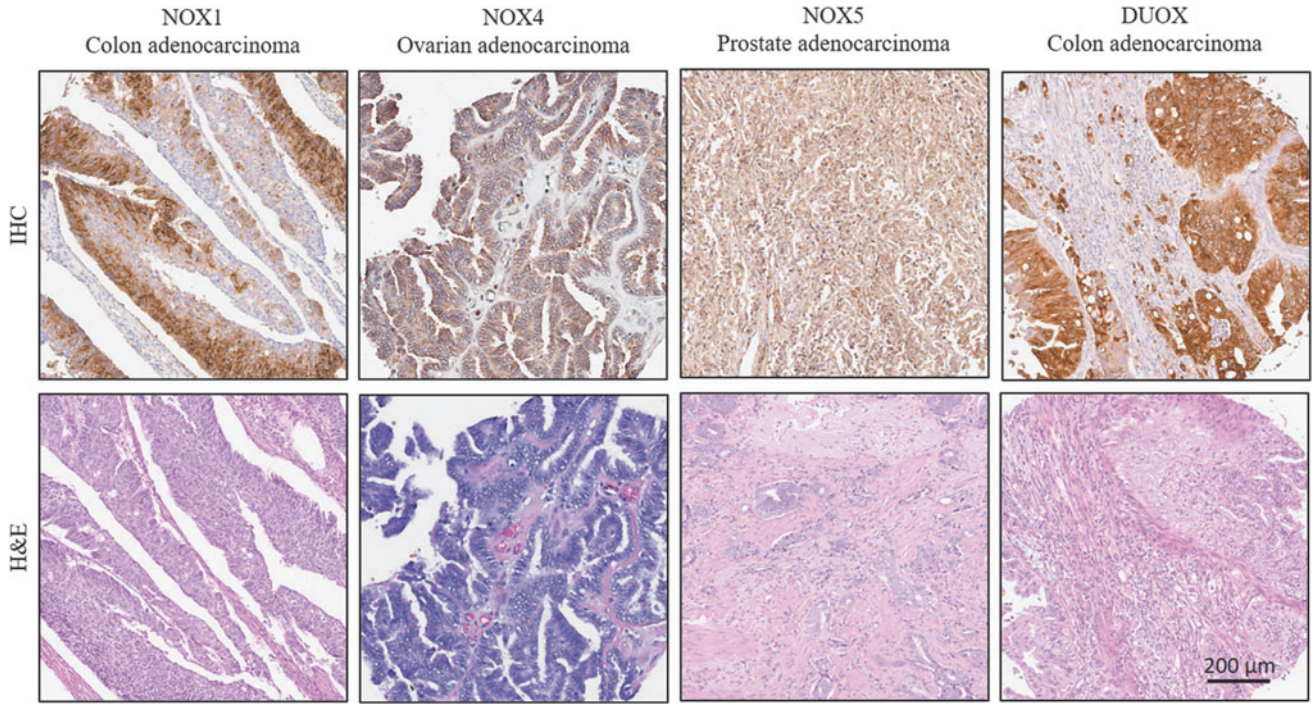


FIG. 1. Immunohistochemical detection of NOXs in human tumors. Representative images showing positive IHC staining of NOX1 in colon adenocarcinoma, NOX4 in serous papillary ovarian adenocarcinoma, NOX5 in prostate adenocarcinoma, and DUOX in colon adenocarcinoma. The NOX1, NOX4, NOX5, and DUOX antibodies used for the IHC were developed by our group [(8, 129, 189) and unpublished results]. Tissues are from BioMax multi-tumor tissue arrays (BC05002 and BC051110a for the colon adenocarcinoma tissues, MC6163 for the ovarian adenocarcinoma, and PR2085b for the prostate adenocarcinoma). Images were taken at 10×digital magnification. DUOX, dual oxidase; IHC, immunohistochemical; NOX, NADPH oxidase.

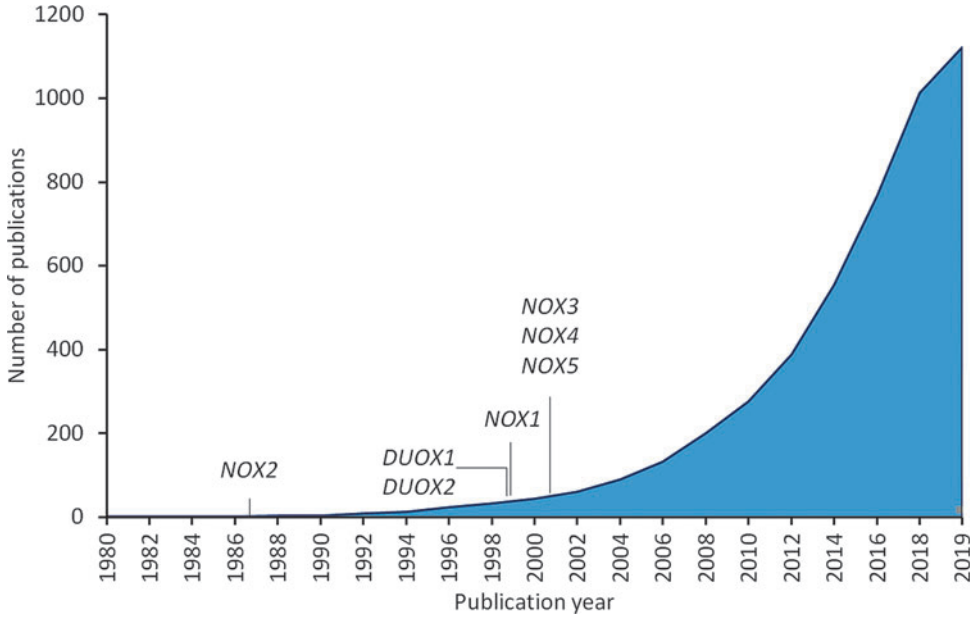


FIG. 2. Cumulative number of publications referencing the terms "NADPH oxidase" and "cancer" in PubMed from 1980 to the present day. The discovery and first report of cloning is indicated for the various NOX isoforms (19, 20, 38, 50, 55, 60, 98, 148, 159, 166, 171).

distinctive ways (28). Using small hairpin RNA (shRNA) technology to knock down NOX1, de Carvalho *et al.* demonstrated a clear role for NOX1 in $O_2^{\bullet-}$ production and proliferation of colon carcinoma cell lines (49). These results were further supported by a 2012 study by Kajla *et al.* (96a) wherein NOX1-mediated Wnt signaling induced cell growth and RAS transformation. Additional genetic knockdown studies detailing mechanisms through which NOX1-mediated ROS affect the proliferation of HT-29 and Caco-2 human colon cancer cells revealed that knockdown of NOX1 led to cell cycle arrest or apoptosis, depending on the cell line studied and extent of NOX1 knockdown (183). Wang *et al.* observed a significant decrease in the growth of HT-29-shNOX1 cells, which occurred, in part, by way of the ADAM17-EGFR-PI3K-Akt signaling pathway (181). Similarly, our laboratory found that silencing NOX1 inhibited the growth of HT-29 cells, and this effect was associated with the downregulation of several oncogenes, chemokines, and angiogenic factors (95). The significant decrease in tumor growth and angiogenesis on NOX1 attenuation was corroborated by *in vivo* studies from parental HT-29 cells *versus* NOX1 knockdown cell xenografts (95).

NOX2 expression has been associated with the growth of breast, colorectal, gastric, and prostate cancers, and with myelomonocytic leukemia (78, 81, 85, 100, 132). In breast cancer cells, silencing of the superoxide-generating NOX2 resulted in significant reduction of IKKε (inhibitor of NF-κB kinase) expression, a key player in cell transformation, invasiveness, and the development of chemoresistance (132). In gastric cancer, the small interfering RNA (siRNA)-mediated downregulation of NOX2 decreased cell viability and ROS content (100).

Several NOX4 genetic knockdown studies in various tumor types have been reported. In non-small cell lung cancer (NSCLC) cell lines, the depletion of NOX4 inhibited NSCLC cell aggressiveness and NOX4 overexpression-mediated metabolic effects. In gastric cancer, RNA interference (RNAi)-mediated silencing of NOX4 inhibited cell adhesion and invasive potential of gastric cancer cells through JAK2/STAT3 signaling (73). In human neuroblastoma cells, silencing of NOX4 suppressed glycolysis induced by hypoxia and cell growth through inhibition of the PI3K/Akt signaling pathway (198). One major weakness of many genetic knockdown studies, however, is the absence of a direct linkage between decreased gene expression and NOX-related production of ROS, limiting our understanding of the functional consequences of NOX inhibition.

Höll *et al.*, using shRNA-mediated knockdown of NOX5 in NOX5-expressing (PC-3, LNCaP) but not NOX5-negative (DU145) prostate cancer cell lines, demonstrated that NOX5-derived ROS and the subsequent depletion of PKCζ and inaction of JNK played a critical role in the proliferation and survival of prostate cancer cells (86). The suppression of NOX5 expression produced a significant impact on the growth and proliferation of different tumor cell types in multiple studies, including in Barrett's esophageal adenocarcinoma cells involving cyclooxygenase 2, p16, silencer-of-death domain (SODD) and NF-κB regulation (113, 161), breast cancer cells *via* STAT5A-mediated NOX5-L expression (52), malignant melanoma involving pathways regulating HIF-1α and networks that signal through Akt/GSK3β/$p27^{Kip1}$ (7), ALK-positive anaplastic large-cell lymphoma cell lines where NOX5-derived ROS contribute to apoptosis blockage (34), and adult T cell leukemia (ATL) where NOX5α was key to maintaining HTLV-1 transformation phenotype (157). In addition, depending on expression level and cellular context, NOX5 may play a dual role in cancer cells; NOX5 was shown to promote cell death *via* Ca^{2+} and c-ABL, while also being able to stimulate tumor growth in some cancers *via* STAT5A and CREB. Thus, NOX5 may determine the balance of cellular proliferation and death in skin, breast, and lung cancer cells (51).

The epigenetic silencing of DUOX1 has been reported in lung and liver cancers, suggesting a tumor suppressor role for DUOX1 in these diseases (117, 123). Stably knocking down the expression of DUOX1 in nontumor mammary cells

(MCF12A) with shRNA led to higher cell proliferation rates and decreased migration and adhesion properties, suggesting that breast carcinogenesis may involve the silencing of DUOX1 (67). However, in contrast to its postulated tumor-suppressing ability in lung, liver, and breast cancer, DUOX1 has also been implicated in the promotion of genomic instability and radiation-induced thyroid tumorigenesis, as the downregulation of DUOX1 with a specific RNA abrogated the induction of DNA damage after ionizing radiation (6).

The expression of DUOX2 in human tumors has been associated with long-standing chronic inflammation. In pancreatic ductal adenocarcinoma cells, immunomodulatory and proinflammatory cytokines and bacterial cell wall component lipopolysaccharide (LPS) specifically upregulated DUOX2 and its maturation factor DUOXA2 (dual oxidase 2 maturation factor). Our group recently demonstrated the ability of inflammatory mediators such as interferon-gamma (IFN-γ), LPS, IL-4, and IL-17A to upregulate DUOX2 and increase the accumulation of H_2O_2 and DNA damage through STAT6 and NF-κB signaling, effects that could be suppressed with the NOX inhibitor diphenylene iodonium (DPI) (191, 193). In hepatocellular carcinoma (HCC), the depletion of DUOX2 abrogated PKCα-induced activation of Akt/MAPK signaling pathways as well as cell proliferation, migration, and invasion in HCC cells (182). These reports demonstrate that, depending on the cell context, DUOX-derived H_2O_2 might have tumor-promoting activities, but under some conditions the accumulation of DUOX-associated ROS may lead to growth arrest and senescence, thereby contributing to tumor suppression.

NOX and the malignant phenotype

NOX activity is induced by stimuli such as growth factors, cytokines, or calcium fluxes (127, 147). Excessive ROS produced by NOX proteins promote a pro-oxidative cellular environment that fosters genomic instability and cellular transformation due to the accumulation of macromolecular damage (24, 25, 39). Further, $O_2^{\bullet-}$ and H_2O_2 were found to contribute to multiple signal transduction pathways, in part through the activation of protein kinase and cytokine signaling (29, 106, 162). For instance, the increased glycolytic activity often observed in cancer cells, also known as the Warburg effect, that allows tumor cells to proliferate in spite of defective mitochondrial respiration caused by hypoxia or mitochondrial injury, can be supported by the NOX-dependent restoration of NAD^+ stores, as reported by Lu *et al.*; and NOX-associated ROS may also promote the HIF-1α-dependent expression of glycolytic genes (46, 122, 195). A recent study by Bertram *et al.* demonstrating the involvement of NOX1 in the metabolic remodeling of hepatoblastoma cells toward a more proliferative state further supports this notion (23).

A plethora of studies have implicated NOX1, NOX2, NOX4, and DUOX2 as triggers of angiogenesis and promoters of invasiveness *via* the ERK-dependent upregulation of vascular endothelial growth factor (VEGF) and matrix metalloproteinases, and the activation of other redox-sensitive transcription factors such as HIF-1α, TP53, and NF-κB (11, 36, 74, 95, 128, 129, 175, 193, 194). A role for NOX1-generated ROS in cell invasion was reported in the context of metalloprotease production and cell motility, where the augmented invasiveness of KRAS-transformed normal rat kidney cells and epidermal growth factor-stimulated migration of Caco-2 cells was attenuated by transfection of NOX1 siRNAs (158). DUOX2 knockdown with siRNA significantly decreased IFN-γ-induced VEGF-A or HIF-1α upregulation in pancreatic cancer cells (193).

Increased tumor angiogenesis is associated with epithelial–mesenchymal transition (EMT), a process that facilitates tumor initiation, stemness, and resistance to anticancer therapy (65, 135). In fact, the activity of H_2O_2-producing enzymes NOX4 and DUOX2 in cancer cells was linked to EMT, a process that confers tumor cells with a phenotypic plasticity that allows for therapy evasion (84, 97, 110, 128). In breast cancer cells, NOX4 knockdown blunted TGF-β-induced wound healing and cell migration, key steps in TGF-β and SMAD3-driven induction of the epithelial-to-mesenchymal transition and migration of breast epithelial cells (26). In human gastric cancer cells, the downregulation of DUOX2 increased radioresistance; whereas in colon cancer cells, the expression of DUOX2 promoted chemoresistance and aggressiveness *via* the TET1/DUOX2/ROS/EMT axis (97, 136). In addition, in prostate carcinoma (PCa), inhibition of stromal NOX4 abrogated the enhanced proliferation and migration of PCa cell lines induced by TGF-β1-activated prostate fibroblast conditioned media (149). In ovarian cancer cells, knockdown of either the low-affinity leukotriene B_4 receptor BLT2 or NOX4 using specific siRNAs suppressed STAT3 stimulation and MMP2 expression, leading to attenuated invasiveness (155). Further, NOX1-, NOX2-, and NOX4-derived ROS increase resistance to apoptosis in cancers of the liver, bladder, and gastrointestinal tract (109, 112, 146, 152, 160, 177, 186). In contrast, RNAi-mediated DUOX1 silencing in NCI-H292 epithelial lung cancer cells promoted EMT, cancer stem cell characteristics, and invasive properties (118). Immunosuppression *via* inhibition of T cell responses, as reported for NOX2 in multiple studies, constitutes another avenue by which NOX proteins can mediate tumor growth (35, 37, 43, 186).

NOX protein expression in human malignancies

In line with the earlier findings linking NOX activity to tumor development and progression in preclinical models of cancer, overexpression of NOX1 has been observed in tissue specimens taken from patients with premalignant gastrointestinal inflammation or with colon adenocarcinoma (14, 33, 95, 107, 119, 169). In data from the PanCancer Atlas study of The Cancer Genome Atlas (TCGA), messenger RNA (mRNA) expression of NOX1 was notably higher in colorectal adenocarcinoma than in any other cancer types (Fig. 3). In addition, modest NOX1 mRNA levels are present in gastric and esophageal adenocarcinomas. Correspondingly, high levels of NOX1 expression may promote DNA breaks, base (guanine) oxidation, and general genomic instability; all of which are cellular events that are consistent with tumorigenesis (39). Juhasz *et al.* have provided a biological link between NOX1 expression and the pathogenesis of colon cancer by demonstrating the pro-proliferative effect of NOX1-associated ROS in colon cancer cells (95). Further, inhibition of NOX1, either genetically or chemically, was sufficient to prevent the onset of premalignant inflammatory states and to slow the growth of NOX1-containing murine tumors (74).

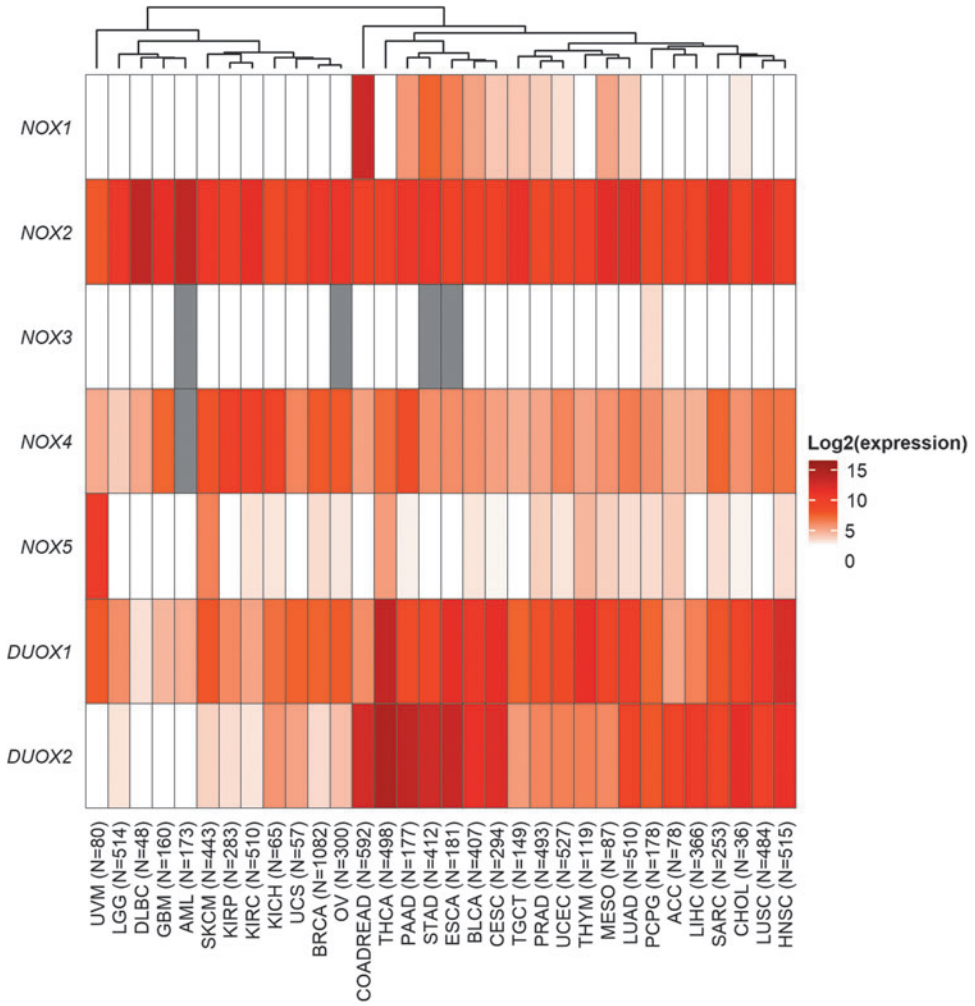


FIG. 3. Landscape of NOX protein expression in human cancer specimens from TCGA. Hierarchical clustering on log-transformed mean gene expression values in 32 cohorts of TCGA (TCGA Research Network). *Columns* were clustered by using Euclidian distance and complex linkage. mRNA expression data (batch-normalized read counts from Illumina HiSeq_RNASeqV2) was downloaded from cBioPortal (72). *Red*: higher expression; *white*: lower expression; *gray*: missing value. The number of specimens per cohort is indicated in *parentheses*. ACC, adrenocortical carcinoma; AML, acute myeloid leukemia; BLCA, bladder urothelial carcinoma; BRCA, breast invasive carcinoma; CESC, cervical squamous cell carcinoma; CHOL, cholangiocarcinoma; COADREAD, colorectal adenocarcinoma; DLBC, diffuse large B cell lymphoma; ESCA, esophageal adenocarcinoma; GBM, glioblastoma multiforme; HNSC, head and neck squamous cell carcinoma; KICH, choromophobe renal cell carcinoma; KIRC, renal clear cell carcinoma; KIRP, renal papillary cell carcinoma; LGG, brain lower grade glioma; LIHC, liver hepatocellular carcinoma; LUAD, lung adenocarcinoma; LUSC, lung squamous cell carcinoma; MESO, mesothelioma; mRNA, messenger RNA; OV, ovarian serous cystadenocarcinoma; PAAD, pancreatic adenocarcinoma; PCPG, pheochromocytoma and paraganglioma; PRAD, prostate adenocarcinoma; SARC, sarcoma; SKCM, skin cutaneous melanoma; STAD, stomach adenocarcinoma; TCGA, The Cancer Genome Atlas; TGCT, testicular germ cell tumors; THCA, thyroid carcinoma; THYM, thymoma; UCEC, uterine corpus endometrial carcinoma; UCS, uterine carcinosarcoma; UVM, uveal melanoma.

In addition to its expression in phagocytes, NOX2 is highly expressed in hematopoietic malignancies (1, 13, 85, 94, 125). NOX2 can also be found in immune cells that infiltrate tumors, indicative of tissue inflammation. Correspondingly, in the TCGA-PanCancer Atlas study, NOX2 mRNA expression is high in the majority of cancer cohorts, including diffuse large B cell lymphoma (DLBC cohort), acute myeloid leukemia (AML), breast invasive carcinoma, and lung adenocarcinoma (Fig. 3). In marked contrast, to date, NOX3 has not been linked to any malignancy, in accordance with the lack of NOX3 mRNA expression in TCGA cohorts (Fig. 3).

NOX4, which is usually present in the kidney and in endothelial cells, has also been detected in multiple solid tumors, including malignancies of the brain, skin, kidney, and ovary (103, 129, 159, 160). NOX4 has also been implicated in TGF-β-induced EMT, a process that has been linked to the acquisition of chemoresistance (26, 84). In TCGA cohorts, the highest mRNA expression of NOX4 is observed in three kidney cancer cohorts: renal papillary cell carcinoma (KIRP), renal clear cell carcinoma (KIRC), and chromophobe renal cell carcinoma (KICH) (Fig. 3). Further, specimens from pancreatic adenocarcinoma, invasive breast carcinoma,

Table 1. Tissue Distribution of NADPH Oxidase Enzymes in Normal Tissues and Malignant Diseases

Enzyme	*Normal tissue distribution*	*Associated malignancies*
NOX1	Colon epithelium (166)	Colorectal adenocarcinoma (14, 33, 39, 95, 119, 169)
NOX2	Phagocytes (17)	Hematopoietic and lymphoid cancers (1, 13, 85, 125)
NOX3	Inner ear (18)	N/A
NOX4	Kidney, endothelial cells (75, 103, 159)	Kidney, ovary, brain, and bladder cancer; melanoma (103, 129, 159, 160)
NOX5	Spleen, testis, and lymphoid tissue (20)	Prostate cancer, malignant melanoma (7, 86)
DUOX1	Thyroid, airway epithelium, and prostate (30, 50, 60, 76, 180)	Thyroid and head and neck cancer; thymoma
DUOX2	Thyroid, salivary gland, rectal mucosa, gastrointestinal tract, airway epithelium, and prostate (30, 50, 60, 63, 76, 180)	Thyroid, breast, colorectal, gastric, lung, prostate, and pancreatic cancer (69, 141, 189, 190, 192, 193)

DUOX, dual oxidase; NOX, NADPH oxidase.

ovarian serous cystadenocarcinoma, glioblastoma, and melanoma moderately express NOX4, in line with published reports (103, 129, 159, 160).

NOX5 has no orthologs in rodent genomes, thus rendering the study of NOX5 in preclinical cancer models difficult. NOX5 has been implicated in prostate cancer due to its reported expression in PC-3 prostate cancer cells (88). The recent development of a validated NOX5 antibody has allowed the immunodetection of the protein in UACC-257 malignant melanoma cells, as well as in multiple human tumors, including ovarian, prostate, and lung cancers, in tissue microarrays (8). In TCGA clinical specimens, NOX5 mRNA is highly expressed in uveal and skin melanoma, and to a lesser extent in thyroid carcinoma, as can be appreciated from the heatmap in Figure 3.

Both DUOX1 and DUOX2 are associated with normal thyroid function, as well as with host defense activities in airway epithelium and the gastrointestinal tract (30, 50, 60, 76). DUOX1 is frequently epigenetically silenced in lung and liver cancer, and it has generally low expression in other cancers (94, 117, 118, 123); however, DUOX1 appears to have moderate to high expression in a large fraction of TCGA cohorts, possibly related to its role in host defense (Fig. 3). DUOX2 has been shown to be overexpressed in inflammatory bowel disease, pancreatitis, and in breast, colorectal, gastric, lung, prostate, and pancreatic cancer compared with normal adjacent tissue (69, 141, 189–193). Bioinformatic analysis of TCGA cancer cohorts reveals high mRNA expression of DUOX2 in thyroid carcinoma, colorectal, pancreatic, stomach, and esophageal adenocarcinoma; moderate expression levels are observed in cholangiocarcinoma, head and neck squamous cell, and HCC (Fig. 3).

The tissue distribution of NOX proteins in normal tissue and in disease is summarized (22, 28, 94) and depicted in Table 1. Taken together, these results and observations confirm that NOX proteins constitute potential therapeutic targets for cancer treatment.

Prognostic value of NOX mRNA expression levels in cancer

Recent studies have leveraged the large collection of genomic data made available for research through TCGA to evaluate the prognostic potential of NOX mRNA expression in colorectal and gastric cancer, as well as in HCC. When comparing 458 colorectal cancer specimens with 41 normal samples, Cho *et al.* found the NOX family to be generally overexpressed at the mRNA level in tumor relative to normal tissue, and NOX4 and NOX5 to be associated with poor prognosis, whereas NOX1 and DUOX2 were associated with better prognosis (40). Eun *et al.* reported that NOX1, NOX2, and NOX5 were associated with poor prognosis in patients with HCC, in contrast to NOX4 and DUOX1 that were associated with better survival in these patients (64). You *et al.* reported the overexpression of NOX1, NOX2, and NOX4 in gastric cancer specimens, and they suggested that NOX4 and DUOX1 expression had a negative impact on the survival of these patients (197). Finally, we reported that DUOX2 mRNA expression was indicative of poor survival in the pancreatic adenocarcinoma patient cohort from TCGA (191). These results are by no means comprehensive, as TCGA comprises more than 30 cohorts spanning different malignancies. Further, the limits of using mRNA rather than protein expression for prognostication must be acknowledged. The variable and context-specific nature of these prognostic associations highlights potential challenges in applying NOX inhibition in the clinic for the treatment of cancer.

Genetic Inhibition of NOX in Preclinical Cancer Models

Physiological and pathophysiological functions of NOX members can be explored by using the genetically engineered mouse model (GEM) and/or the NOX-deficient mouse model. The NOX5 gene is absent from rodent genomes, and therefore cannot be studied by using knockout murine models.

Garrido-Urbani *et al.* discovered in 2011 that mice deficient in NOX1 have impaired angiogenesis. Silencing of NOX1 decreased endothelial cell migration and tube-like structure formation through the inhibition of PPARα, a regulator of the NF-κB signaling pathway (74). Further, Stalin *et al.* have recently reported the effect of the genetic deletion of NOX1 in preclinical immunocompetent mouse models of colorectal cancer and melanoma. In these experiments, NOX1 inhibition had potent antitumor effects: These effects were dependent on an intact immune system and required the inhibition of host NOX1 (163). Moreover, in GEM models of hepatocarcinogenesis, NOX1$^{-/-}$ mice developed 80% fewer tumors, which were 50% smaller than those of wild-type (WT) mice. Interestingly, the ablation of NOX1 in macrophages dramatically abolished the NOX1-mediated development of

HCC by dampening the production of inflammatory cytokines in macrophages (115). NOX1-inhibitory effects have also been observed after the inhibition of NOX accessory proteins. In the K19-C2mE murine model of gastric cancer, disruption of NADPH oxidase organizer 1 (NOXO1), a component of the functional NOX1 enzymatic complex, significantly suppressed gastritis-associated metaplastic hyperplasia (61). In addition, a novel $p47^{phox}$-dependent regulatory mechanism, shifting the immune balance toward colon cancer, was demonstrated when $p47^{phox-/-}$ mice defective in the IL-23/IL-17 axis were protected from colon cancer (145).

The influence of NOX2-derived $O_2^{\bullet-}$ and its oxidative metabolites on the acquisition of the metastatic phenotype using $gp91^{phox-/-}$ mice was initially reported by Okada *et al.* in 2006 (138). This effect of NOX2 on metastasis was further explored in genetically engineered NOX2-deficient mice using melanoma cells. The formation of lung metastases was reduced in B6.129S6-$Cybb^{tm1DinK}$ (NOX2-KO) *versus* NOX2-sufficient WT mice, in part due to a restored IFN-γ-dependent, NK cell-mediated clearance of melanoma cells (16). A similar decrease in pulmonary metastatic colonization was observed in mice deficient for all the individual subunits of NOX2 (encoded by CYBA, CYBB, NCF1, NCF2, and NCF4); however, the CYBA-deficient ($Cyba^{tm1a}$) mice showed the most significant decrease in metastatic colonization. NOX2 deficiency resulted in granulomas and in the accumulation of antitumoral immune cells in the lungs (176). In murine models of BCR-$ABL1^+$ leukemia and a syngeneic, orthotopic model of prostate cancer, genetic ablation of NOX2 reduced the *in vivo* expansion of murine BCR-$ABL1^+$ hematopoietic cells, and it caused a reduction in tumor growth and angiogenesis in the murine prostate cancer model $NOX2^{-/y}$ (78, 81). Recent reports have also demonstrated that NOX2-derived ROS contribute to the progression of KRAS-induced leukemia, in a series of experiments wherein the expansion of leukemia was markedly reduced in triple transgenic mice with genetic NOX2 deficiency ($NOX2^{-/-} \times$ LSL-$Kras^{G12D} \times$ Mx1-Cre) that expressed KRAS in their hematopoietic cells (15, 125).

The pro-angiogenic function of NOX4 was also investigated *in vivo*, notably with a model of chemically induced carcinogenesis in mice. Tumor angiogenesis induced by the carcinogen 3-methylcholanthrene (MCA) was significantly reduced, as indicated by the observed reduction in tumor vascularization and measured defects in hypoxia signaling in $NOX4^{-/-}$ mice compared with the WT, due, in part, to the decreased expression of HIF-1α and VEGF (82). Although additional *in vivo* studies are needed to unequivocally link NOX-mediated ROS to cancer, these results demonstrate that the inhibition of tumor growth through selective targeting of NOXs is achievable *in vivo*, due to the contribution of NOX-associated ROS in the regulation of cell division, survival, and angiogenesis in cancer. We will next consider the extent to which these antiproliferative effects can be recapitulated with the putative small-molecule NOX inhibitors that currently populate the field.

Small-Molecule Inhibitors of NOX Proteins

Unspecific NOX inhibitors

Small-molecule NOX inhibitors can be divided into three categories: ROS scavengers/antioxidants, inhibitors of NOX functional complex formation, and true enzyme antagonists (binding to one of the catalytic domains of the protein to suppress electron transfer). A short list of notable NOX inhibitory molecules is displayed in Table 2. Several anti-inflammatory molecules were discovered in the context of stroke prevention (99). Apocynin, or 4-hydroxy-3-methoxy-acetophenone, is a naturally occurring phenol derived from plants that inhibits inflammation *via* a dual mechanism, by preventing the assembly of an active NOX complex and in a myeloperoxidase-dependent manner, or by exerting nonspecific antioxidant effects (83, 164, 168). The IC_{50} (concentration with 50% inhibition of activity) of apocynin for the inhibition of the phagocytic respiratory burst was measured to be 10 μM. Suzuki *et al.* demonstrated that apocynin had antiproliferative effects in cellular and murine models of prostate cancer; apocynin suppressed the growth and metastasis of PLS10 prostate cancer xenografts in a dose-dependent manner (168). In this study, the antiproliferative effects of apocynin were attributed to the drug-induced dephosphorylation of NOX-complex protein RAC1 and the downregulation of cyclin D1, further confirming that NOXs are not direct targets of this agent (2, 168).

AEBSF, or 4-(2-aminoethyl)-benzenesulfonyl fluoride, is an irreversible serine protease inhibitor that was found to also inhibit NOX2 activation and $O_2^{\bullet-}$ production. Diatchuk *et al.* hypothesized that AEBSF may have a dual mode of action; the charged aminoethyl moiety mimics a basic amino acid, possibly binding to an acidic domain of the NOX complex, whereas the sulfonyl fluoride group may covalently and irreversibly bind to NOX and preclude binding of accessory proteins $p47^{phox}$ and/or $p67^{phox}$ (53). In a study by Wartenberg *et al.*, AEBSF significantly inhibited ROS production in NOX1-overexpressing DU145 prostate tumor spheroids (185). Potency of AEBSF for NOX inhibition is lacking, however, with an $IC_{50} \sim 1$ mM for the inhibition of $O_2^{\bullet-}$ production in intact macrophages (53).

Plumbagin, a plant-derived naphthoquinone, is another unspecific antioxidant molecule that exerts pleiotropic effects (56, 139). Although Ding *et al.* demonstrated the specificity of plumbagin for NOX4 using NOX4-expressing HEK293 and LN229 cells, and NOX4-transfected COS-7 cells, the exact mechanism of action of the molecule remains undefined. Several groups have reported on the antiproliferative effects of plumbagin, both *in vitro* and *in vivo*, mediated by the upregulation of p53 and p21, and the suppression of cell cycle regulators (87, 102, 200). A 2018 study by Du *et al.* described the plumbagin-induced growth suppression of NOX4-expressing, anoikis-resistant MKN45-AR xenografts (59).

Novel NOX inhibitors discovered through functional screens

Large-scale drug screening efforts have fostered the discovery of novel, more selective NOX inhibitors. The most notable inhibitors are listed in Table 2. Through a screen for the discovery of NOX2 inhibitors, the company Vasopharm GmbH discovered the triazolo pyrimidines VAS2870 and VAS3947. These molecules prevented the assembly and functional activation of NOX complexes (172, 188). VAS2870 was shown to inhibit the growth of human HCC cells, presumably through NOX inhibition (153). However,

Table 2. Summary of Notable NADPH Oxidase Inhibitors

Inhibitor	*Molecular target(s), mechanism of action*	*Activity in 3D or murine models of cancer*	*Potency (IC_{50})*	*Literature references*
DPI	Flavoprotein inhibitor that inhibits all NOXs	Inhibited the growth of NOX1-expressing HT-29 and LS-174 colon cancer xenografts	Nanomolar range; see Figure 5B	Doroshow *et al.* (57); Lu *et al.* (121)
DPI analogs	Flavoprotein inhibitors with improved selectivity for NOX isoforms		Nanomolar range; see Figure 5B	Lu *et al.* (121)
Apocynin	Antioxidant and inhibitor or NOX complex assembly	Dose-dependent suppression of tumor growth and metastasis in PLS10 mouse model of prostate cancer	~10 μM (NOX2)	Suzuki *et al.* (168)
AEBSF	Serine protease inhibitor hypothesized to inhibit NOX complex assembly	Inhibition of ROS production in DU145 prostate tumor spheroids	~10 mM (NOX2)	Wartenberg *et al.* (185); Diatchuk *et al.* (53)
Plumbagin	Antioxidant; anti-NOX4 activity	Inhibited the progression of gastric cancer in mice bearing anoikis-resistant MKN-45-AR xenografts	2 μM (NOX4)	Du *et al.* (59); Ding *et al.* (56)
VAS2870, VAS3947	Inhibitor of NOX complex assembly and activation; suppress PDGFR-dependent NOX activation		VAS2870: 2 μM (NOX2); VAS3947: 1–2 μM (NOX2)	Sancho and Fabregat (153); ten Freyhaus *et al.* (172); Wind *et al.* (187)
GKT136901, GKT137831	Specificity for NOX1 and NOX4; possible ROS scavengers	GKT136901 significantly attenuated the growth and vascularization of LLC1 Lewis lung carcinoma xenografts; GKT137831 slowed disease development in an FLT3ITD-transformed AML mouse model	GKT136901: 160–170 nM (NOX1/NOX4); 450 nM (NOX5); GKT137831: 110 nM (NOX1), 140 nM (NOX4), and 410 nM (NOX5)	Laleu *et al.* (104); Musset *et al.* (133); Garrido-Urbani *et al.* (74); Jayavelu *et al.* (92); Gaggini *et al.* (70)
ML171	Selective NOX1 inhibitor; possibility of ROS-assay interference		250 nM (NOX1)	Gianni *et al.* (77)
NOS31	Selective NOX1 inhibitor		2 μM (NOX1)	Yamamoto *et al.* (196)
GLX351322, GLX7013114, and GLX481372	Selective NOX4, NOX5 inhibitors		GLX351322: 5 μM (NOX4); GLX7013114: 0.3 μM (NOX4); GLX481372: ~0.6 μM (NOX4/NOX5)	Anvari *et al.* (9); Wang *et al.* (184)
GKT771	NOX1	Inhibited the growth of MC38 colon carcinoma and B16F10 melanoma tumors in mouse models	K_i = 60 nM (NOX1)	Stalin *et al.* (163)
Nox2ds-tat (peptide)	NOX2 inhibitory peptide; binds $p47^{phox}$-$p22^{phox}$ interface	Inhibited ROS production and vascular tissue damage	740 nM (NOX2)	Jacobson *et al.* (90); Liu *et al.* (120); Csanyi *et al.* (44)
NoxA1ds (peptide)	Disrupts the interaction between NOX1 and NOXA1		20 nM (NOX1)	Ranayhossaini *et al.* (143)
NF02 (peptide)	NOX1 inhibitory peptide		63 μM (NOX1)	Mousslim *et al.* (131)

AEBSF, 4-(2-aminoethyl)-benzenesulphonyl fluoride; AML, acute myeloid leukemia; DPI, diphenylene iodonium; IC_{50}, concentration with 50% inhibition of activity; K_i, inhibition constant; ML171, 2-acetylphenothiazine; ROS, reactive oxygen species.

off-target effects have been reported, and these molecules have poor solubility and pharmacokinetic properties (5, 167).

The company GenKyoTex employed a structure–activity relationship screening approach to develop the pyrazolopyridine dione derivatives GKT136901 and GKT137831, two compounds with selective potency for the inhibition of NOX1 and NOX4, and to a lesser extent, NOX5 (10, 104, 133). GKT136901 significantly attenuated the growth and vascularization of LLC1 Lewis lung carcinoma xenografts, and GKT137831 slowed disease development in an FLT3ITD-transformed AML mouse model (74, 92). These compounds have favorable pharmacological properties, and GKT137831 is currently being evaluated in a clinical trial for patients with idiopathic pulmonary fibrosis (NCT03865927), a condition associated with NOX4-derived ROS (91, 93). Possible caveats to the use of these compounds as NOX inhibitors are the fact that their mechanism of action is unknown, and GKT136901 has been shown to function as an ROS scavenger (154). Moreover, Augsburger *et al.* have recently unequivocally established that the effect of both GKT136901 and GKT137831 was independent of NOX expression, and that the low K_i (inhibition constant) values for NOX inhibition are, in fact, attributable to nonspecific inhibition of the horseradish peroxidase enzyme used in the Amplex Red assay (12).

ML171 (2-acetylphenothiazine) is another putative NOX inhibitor with no known mechanism of action. Its discovery resulted from a large-throughput structure activity relationship screen of commercially available phenothiazine compounds for NOX1 inhibitors conducted by the Scripps Institute (77). ML171 effectively inhibited the ROS-dependent formation of extracellular matrix degrading invadopodia in DLD1 colon cancer cells (77). However, similar to other phenothiazine compounds, ML171 is also a weak inhibitor of serotonin and adrenergic receptors, thus opening the possibility of confounding off-target effects independent of NOX inhibition. Moreover, Seredenina *et al.* determined that ML171 actually interfered with peroxidase-dependent ROS-measuring assays rather than truly inhibiting NOXs (156).

Yamamoto *et al.* identified the microbial metabolite NOS31 produced by *Streptomyces* sp. as a low micromolar inhibitor of NOX1-associated PMA-stimulated production of ROS in colon cancer cells (196). NOS31 lacked both antioxidant and cytotoxic activities and appeared selective for NOX1 ($IC_{50} \sim 2\ \mu M$ compared with ~ 30 to $>40\ \mu M$ for other NOX isoforms). The proliferation of colon and stomach cancer cell lines was inhibited by this compound; however, the authors noted that the mechanism of NOX inhibition by NOS31 remains to be determined. To our knowledge, reports on NOS31 activity *in vivo* have not yet been published.

Wang *et al.* described the discovery and characterization, in collaboration with Glucox Biotech AB, of novel and selective inhibitors GLX351322, GLX7013114, and GLX481372 specific for NOX4 and NOX4/NOX5, respectively, with $IC_{50} < 1\ \mu M$ (9, 184). The discovery of these compounds originated from a high-throughput screen of 40,000 chemicals by using an Amplex Red assay in a 384-well format. General antioxidant activity was ruled out with the 2,2-diphenyl-1-picrylhydrazyl-hydrate (DPPH) assay. GLX351322 prevented ROS production in pancreatic islet cells induced by high glucose in male C57BL/6 mice, and all three compounds protected islet cells from death induced by NOX4-associated ROS (184). To our knowledge, there are no published reports to date on the activity of these compounds in cancer models.

Finally, compound GKT771 developed by GenKyoTex shows promising antitumor activity in NOX1-expressing systems (163). GKT771 significantly attenuated the growth of xenografted MC38 colon carcinoma and B16F10 melanoma tumors in immunocompetent mice. Moreover, the authors noted a concurrent decrease in angiogenesis and lymphangiogenesis, and increased recruitment of tumor-associated immune cells. These effects were dependent on NOX1 expression, as NOX1-deficient mice did not respond to treatment with GKT771 (163). The authors concluded that GKT771 exerts NOX1-dependent immune-modulatory effects that, when used in combination with immune cell checkpoint inhibitor anti-PD1, further potentiate antitumor activity.

Iodonium class flavin dehydrogenase inhibitors

DPI, a flavoprotein inhibitor, inhibits electron-transporting enzymes, some of which are involved in normal cellular metabolism, including nitric oxide synthase, xanthine oxidase, mitochondrial complex I, and the microsomal cytochrome P450 reductase (2, 114). O'Donnell *et al.* empirically demonstrated that the mechanism by which DPI suppresses the flow of electrons from NADPH to FAD, heme redox centers, and, ultimately, to O_2 may be by covalently binding to FAD, forming stable phenylated-FAD adducts (137). Despite its broad specificity for various flavoproteins, DPI is the most widely used NOX inhibitor (96). In a recent study aimed at evaluating the specificity and potency of NOX inhibitors using human cell lines expressing high levels of NOX isoforms, Augsburger *et al.* have shown that, in contrast to molecules such as apocynin, ebselen, GKT136901, and VAS2870, which lacked true NOX inhibition activity, DPI consistently elicited dose-dependent inhibition of all NOX isoforms in multiple assays (12). A study by our group revealed that DPI and a derivative di-2-thienyliodonium (DTI) inhibited the growth of multiple NOX1-expressing human colon cancer cell lines, suppressed ROS production at submicromolar concentrations, and decreased the growth of HT-29 and LS-174 colon cancer xenografts (57). We hypothesized that NOX inhibition may enhance the activity of protein tyrosine phosphatases (PTP) and inactivate key kinases involved in pro-survival signaling (Fig. 4) (57, 58).

To circumvent off-target toxicities (178) and to allow for cancer cell targeting in spite of mitochondrial dysfunction—as is the case of many tumors that rely on glycolysis for energy production rather than the more efficient process of oxidative phosphorylation active in noncancerous cells (122)—we recently suggested that the specificity and selectivity of DPI could be optimized by modifying the position and identity of functional groups around the benzene rings of DPI, as well as the counter-ion to alter reactivity. We synthesized 36 DPI analogs and screened them for improved solubility, inhibition of cell proliferation, and inhibition of ROS production in different NOX-expressing systems (121). Because it has been demonstrated that mitochondrial metabolism can be inhibited by DPI (114), we further screened selected DPI derivatives for those compounds that did not disrupt normal mitochondrial energy metabolism, as assessed

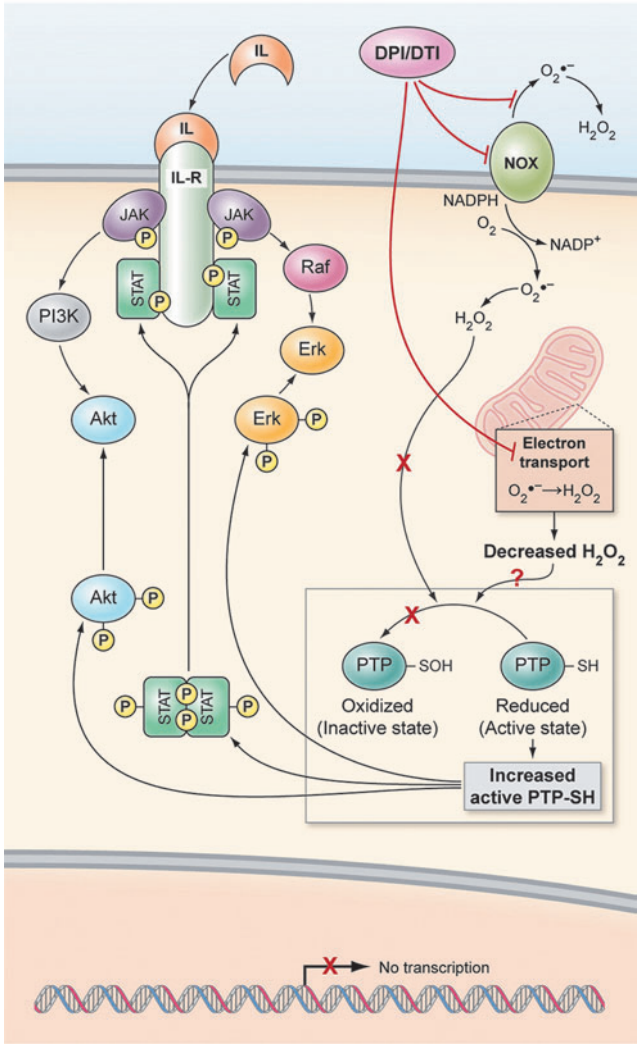


FIG. 4. Hypothetical model of the effect of NOX inhibition on downstream cell signaling. Empirical evidence strongly suggests that NOX-associated ROS promote the activation of JAK/STAT and Akt signaling pathways, and that NOX inhibition with DPI or its analog DTI enhances the activity of intracellular PTP, thus dephosphorylating key signaling kinases. DPI, diphenylene iodonium; DTI, di-2-thienyliodonium; PTP, protein tyrosine phosphatases; ROS, reactive oxygen species. Adapted from Figure 8 in Doroshow *et al*. (58).

through measurements of oxygen consumption rate and extracellular acidification rate (121). This screen uncovered four DPI analogs (National Service Center [NSC] 740104, 751140, 734428, 737392) with potent inhibitory activity against HT-29 cell growth and ROS production (Fig. 5A). All four compounds were more potent than DPI with respect to the selective inhibition of NOX4 and DUOX2; 740104 and 734428 also demonstrated improved potency for NOX1 compared with the parent molecule DPI (Fig. 5B). Further, compounds 734428 and 737392, both of which feature nitro functional groups, had improved selective potency for DUOX2. To further investigate the observation that nitro substitution groups improve compound selectivity for DUOX2, a configurational isomer of NSC 737392 was prepared: NSC 780521 (121). In line with our hypothesis, compound 780521 exhibited a marked selectivity for inhibiting DUOX2-expressing cells, performing markedly better than DPI (Fig. 5B). Moreover, we have shown with a cell-free proof-of-concept experiment that the five novel DPI analogs were capable of covalently binding to reduced FAD to form stable adducts, consistent with O'Donnell *et al*.'s report of DPI binding to FAD as a mechanism of flavoprotein inhibition (137). Mechanistic experiments aimed at further characterizing the effects of these molecules at the cellular level are ongoing. It remains to be determined whether these compounds exert antiproliferative effects by blocking cell cycle progression, as was reported for DPI and DTI (58).

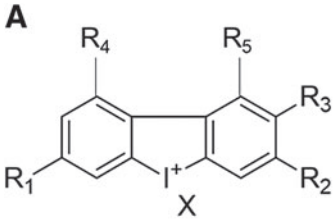
Inhibitory Peptides

To optimize selective targeting of NOX enzymes and decrease risks of off-target toxicity, the development of inhibitory monoclonal antibodies of NOX enzymes has also been explored, although with limited success, and further validation in model systems and against other NOXs to ascertain specificity is needed (4, 31, 199). Because the general domain architecture of NOX enzymes is well defined, and the protein–protein interaction partners that are necessary for functional activation have been extensively described for all NOXs, it follows that selective peptides (>20 residues in length) designed to mimic NOX complex components could be employed to specifically inhibit NOX activity. This approach holds promise for the development of isoform-specific NOX inhibitors (5). NOX2, the first identified NOX protein, has been the focus of most peptide development activities. An extensive review of peptide inhibitors of NOX2 activity corresponding to domains in NOX2, $p47^{phox}$, $p67^{phox}$, and RAC has been published by Dahan and Pick (48). Further, Pagano and colleagues have contributed two peptides with NOX isoform selectivity: Nox2ds-tat, a peptide that mimics the site of interaction between NOX2 and $p47^{phox}$ on the cytosolic B-loop of NOX2; NoxA1ds, which was designed to disrupt the interaction of NOXA1 with NOX1 (42, 44, 143).

The first and best characterized rationally designed NOX inhibitory peptide is Nox2ds-tat. The *tat* sequence, derived from the HIV virus, confers cell-membrane permeability. Isoform selectivity was confirmed in cell-free assays wherein the IC_{50} of NOX2ds-tat for ROS production was measured at 0.74 μM (44). *In vivo* results demonstrated that Nox2ds-tat given intravenously or delivered by adenoviral infection did inhibit ROS production and associated tissue damage (90, 120).

Peptide-based inhibition of NOX4 aimed at targeting the interaction between cytosolic NOX4 B-loop and $p22^{phox}$ N-terminus was unsuccessful, presumably due to the fact that NOX4 and functional activators exist in a constitutively active conformation, making disruption of complex formation difficult to achieve (45, 179). For the inhibition of NOX5-mediated $O_2^{\bullet-}$ production, peptides targeting the regulatory EF-hand Ca^{2+}-binding domain disrupting its interaction with the C-terminal catalytic dehydrogenase domain were designed by Tirone *et al.* (173). No peptide-based inhibitors of NOX3, DUOX1, or DUOX2 have been described in the literature to date, although one could envision devising a peptide targeting the N-terminal peroxidase domain of the DUOX proteins to ensure selectivity for DUOX1/2.

A



	R_1	R_2	R_3	R_4	R_5	X
DPI (NSC 735294)	H	H	H	H	H	Cl^-
104 (NSC 740104)	Br	Br	H	H	H	Br^-
140 (NSC 751140)	CO_2CH_3	H	H	H	H	$4\text{-}CH_3C_6H_4SO_3^-$
392 (NSC 737392)	NO_2	NO_2	H	H	H	Br^-
428 (NSC 734428)	NO_2	H	H	H	H	Cl^-
521 (NSC 780521)	H	H	H	NO_2	NO_2	Br^-

B

	HEK293 NOX1	PMN (NOX2)	UACC-257 (NOX5)	HEK293 NOX4	HEK293 DUOX2/A2
DPI IC_{50} (nM)	233	180	42	880	6,488
Compound	**IC_{50} for DPI analogs relative to IC_{50} for DPI (fold-ratio)**				
104	0.30	2.67	1.05	0.18	0.64
140	1.07	0.98	1.31	0.14	0.31
392	1.53	4.72	4.95	0.16	0.03
428	0.58	1.80	0.83	0.30	0.06
521	4.37	5.83	5.45	6.29	0.12

FIG. 5. IC_{50} of DPI analogs relative to IC_{50} of DPI for inhibition of ROS production in NOX-expressing human cells. Adapted from Lu *et al.* (121). **(A)** Chemical structure of DPI analogs selected from a screen for $O_2^{\bullet-}$ inhibition and antiproliferative effects, and lack of potency for mitochondrial activity. The identity of substitution groups and counter ions for each compound are indicated in the table. **(B)** PMA-induced $O_2^{\bullet-}$ production was measured by luminescence assay in HEK293 NOX1, PMN (NOX2), and UACC-257 (NOX5) cells. H_2O_2 production was measured by Amplex Red assay of DPI or analog-treated NOX4 and DUOX2/DUOXA2 overexpression cell lines. The cells were incubated with DPI analogs for 30 min before assay measurement. IC_{50} (n*M*) for DPI in NOX-expressing cell lines is shown in the *first row*, and fold-ratio IC_{50} for DPI analogs compared with IC_{50} DPI is color coded in the following *rows*. *Green shading* denotes improved potency compared with DPI, and *orange shading* indicates decreased potency compared with DPI. DUOXA2, dual oxidase 2 maturation factor; H_2O_2, hydrogen peroxide; IC_{50}, concentration with 50% inhibition of activity; $O_2^{\bullet-}$, superoxide; PMN, polymorphonuclear leukocytes.

Mousslim *et al.* recently presented NF02, a promising NOX1-specific inhibitory peptide identified through a screen with NOX1-expression HT-29 colon cancer cells (131). The authors demonstrated the lack of scavenger activity and cytotoxicity, as well as the selectivity for NOX1 over other NOX2 and other flavoenzymes. The IC_{50} for inhibition of HT-29 proliferation was $\sim 63\,\mu M$, and cell migration and invasion were significantly suppressed by NF02.

Although the peptide inhibitor approach is attractive due to the inherent specificity of such peptides for precise NOX isoform targeting, there are also limitations to translating these molecules into therapies for the clinic. First, peptides are readily degraded in animal systems and have limited bioavailability. Therefore, screening of peptide libraries for selective NOX inhibitors is mostly supported by cell-free assays (48). New delivery methods, such as nanoparticle formulations, and strategic design optimizing pharmacological properties for peptide-based inhibitors should be devised to optimize inhibitory activity *in vivo* (27, 48, 68, 143). Further, due to the strong conservation of functional domains between NOX proteins, isoform selectivity remains difficult to achieve. For instance, El-Benna *et al.* (62) and Dahan *et al.* (47) have created multiple NOX2-derived inhibitory peptides; however, the most potent peptides targeted the cytosolic FAD- and NADPH-binding domains conserved across all NOX proteins. To date, no peptide-based inhibitors of NOX proteins have entered clinical development.

Challenges and Perspectives

The unavailability of high-resolution full-length crystal structures for NOX isoforms has hindered efforts to develop specific and selective NOX inhibitors (124). Without such inhibitors and due to the lack of precision in assays commonly employed to measure ROS production (54, 79), the true impact of NOX enzymes in cancer remains to be fully elucidated. The use of unspecific NOX inhibitors in research can cause systemic ROS inhibition, having deleterious effects on important processes such as the innate immune system (142). For these reasons, the landscape of NOX inhibitors developed to date mostly comprises molecules having pleiotropic effects on cellular activities and metabolism, so that the reported antitumor effects cannot be unambiguously ascribed to NOX inhibition.

The fact that some studies have reported a pro-apoptotic or tumor-suppressing role for NOX enzymes in some cancers

further complicates the matter (151). The role of NOX4 is complex and context specific. NOX4 is the main mediator of TGF-beta-induced apoptosis in many nontumoral and tumoral cells (150, 151). In addition to its oncogenic role in gastrointestinal cancers (197), NOX4 downregulation and deletion have been reported in HCC and liver cancer cells, and correspondingly, NOX4 expression was found to be associated with better prognosis for HCC patients (64). Similarly, DUOX1 is frequently epigenetically silenced in lung and liver cancers, suggesting a tumor suppressor role for DUOX1 in these diseases (117, 123). NOX5 also has both pro-apoptotic and pro-oncogenic roles in diseases such as skin, breast, and lung cancer (51).

Further, NOX-associated ROS is reported to potentiate cancer drug toxicity *via* ROS overproduction (140). Indeed, many chemotherapeutic agents exert antitumor effects by inducing ROS production and oxidative stress, and NOX1 activity was found to potentiate the activity of genotoxic agents such as paclitaxel and oxaliplatin (3, 41). In the latter case, Chocry *et al.* demonstrated that ROS could impact cell survival in dramatically different ways, depending on the level and duration of production; short-term NOX1 activation induced by oxaliplatin exposure favored cell death, whereas sustained NOX1 activation led to the activation of survival pathways through p38 MAPK signaling (41). In addition, per a recent study by Le Gal *et al.*, antioxidant molecules N-acetylcysteine (NAC) and vitamin E analog Trolox significantly increased the invasiveness of a xenografted malignant melanoma tumor (108). Therefore, NOX inhibition, and more generally, antioxidant therapy, is only desirable in certain specific contexts.

Conclusions

There is ample empirical evidence that ROS promote the development and progression of human cancers. Therefore, it follows that NOX enzymes, which constitute a predominant source of ROS in mammalian cells, are likely to play an important role in tumorigenesis. This premise is supported by the fact that NOX overexpression was detected in specimens from various premalignant inflammatory diseases. Intracellular and extracellular ROS contribute to the biology of cancer by inducing DNA damage and oxidation of other cellular components, inducing the activation of oncogenes, such as RAS and c-Myc, and promoting mitogenic signaling by deactivating protein phosphatases (101, 116). ROS also induce the release of cytokines and chemokines, which, in turn, modify the tumor microenvironment. H_2O_2 has been shown to promote metastasis by inducing EMT—a process that is a feature of embryogenesis and wound healing whereby cell adhesion is disrupted and motility increased—but can be aberrantly activated in adult cells (32). Further, Chen *et al.* have recently described how NOX-associated ROS promote tumor-induced immunosuppression (37). Keeping in mind the caveats mentioned earlier regarding the context-specific properties and function of ROS, gaining a better understanding of the specific role of NOX in cancer and pleiotropic effects of associated ROS will require potent and selective inhibitors of NOX isoforms with favorable pharmacological properties. The rational design approach has already yielded some interesting candidates, although the mechanism of action has not been fully elucidated for some of these compounds. Continued efforts to resolve crystal structures for NOX proteins will allow for further optimization of lead NOX inhibitor compounds for *in vivo* evaluation, and ultimately, clinical development for the treatment of cancer.

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Abbreviations Used

AEBSF = 4-(2-aminoethyl)-benzenesulfonyl fluoride
AML = acute myeloid leukemia
DPI = diphenylene iodonium
DTI = di-2-thienyliodonium
DUOX = dual oxidase
EMT = epithelial–mesenchymal transition
FAD = flavin adenine dinucleotide
GEM = genetically engineered mouse models
H_2O_2 = hydrogen peroxide
HCC = hepatocellular carcinoma
IC_{50} = concentration with 50% inhibition of activity
IFN-γ = interferon-gamma
LPS = lipopolysaccharide
ML171 = 2-acetylphenothiazine
mRNA = messenger RNA
NAD = nicotinamide adenine dinucleotide
NOX = NADPH oxidase
NSCLC = non-small cell lung cancer
$O_2^{\bullet-}$ = superoxide
PCa = prostate carcinoma
RNAi = RNA interference
ROS = reactive oxygen species
shRNA = small hairpin RNA
siRNA = small interfering RNA
TCGA = The Cancer Genome Atlas
VEGF = vascular endothelial growth factor
WT = wild-type