Review Article

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Oxidative Stress induced by Over-Expressed NADPH Oxidase (NOX) Initiates Breast Cancer, Promotes Metastasis via Epithelial to Mesenchymal Transition (EMT), Remodels Chromatin and Confers Drug Resistance.

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ABSTRACT

Nicotinamide adenine dinucleotide phosphate (NADPH) oxidases (NOX) are the known enzymes in the human body that are exclusively involved in the production of reactive oxygen species (ROS). The trans-membrane protein NADPH oxidase is known to contain seven isoforms, NOX1-5 and DUOX1-2, which catalyze the generation of reactive oxygen species (ROS) needed for a variety of cellular functions. Strong anti-oxidant networks are necessary for cells to scavenge ROS and shield biological components from oxidative damage because too much ROS causes oxidative stress. The overexpression of NOX isoforms is typically linked to the carcinogenesis of several types of carcinomas, including breast cancer (BC). Because NOX isoforms are overexpressed in BC, they cause oxidative stress, damage mtDNA, and promote angiogenesis and EMT, which in turn aid in BC spreading. With the exception of BC, a number of NOX inhibitors are being used to treat different diseases. Although numerous researchers have suggested that blocking NOX activity is a viable therapeutic approach for individuals with various types of cancer, the therapeutic utility of this approach has not yet been confirmed by clinical trials. Given that breast cancer is the most frequent cancer globally, it's critical to comprehend how ROS and NOX isoforms contribute to the pathobiology of the disease and investigate the possible therapeutic applications of NOX inhibitors in the treatment of breast cancer. In an effort to validate the role of NOX inhibitors as a successful therapeutic approach to controlling BC, this review paper attempts to give a thorough explanation of the involvement of NOX isoforms in the promotion of metastasis associated with BC.

Keywords : Oxidative stress, Breast Cancer, NADPH, NOX, NOX inhibitors, Epigenetic alterations, anti-oxidant defense system.

INTRODUCTION

Reactive oxygen species (ROS) are transient, highly reactive molecules generated under physiological conditions, acting as a second messengers that modulate many cellular activities, including immune response, cellular signaling pathways, and gene transcription [1-3]. ROS includes oxygen radicals, such as nitric oxide (•NO), alkoxyl, hydroxyl radical (•OH), peroxynitrite (NO3 —), superoxide radical anion (O2•–), and oxygen non-radicals, including ozone (O3), hypochlorous acid (HOCl) ,singlet oxygen ($^1O_2$)), and hydrogen peroxide (H2O2) [4]. From these, singlet oxygen ($^1O_2$) and hydroxy radical (OH^-) are known as highly reactive and can damage DNA [5]. Both exogenous and endogenous sources can generate ROS. Exogenous sources are radiations, pharmaceutical drugs, cigarette smoking, and many others. In contrast, endogenous ROS can be produced by both enzymatic and non-enzymatic reactions. Enzyme-catalyzed reactions that produce ROS are

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regulated by different enzymes, notably xanthine oxidase, NADPH oxidases (NOX), uncoupled endothelial nitric oxide synthase (eNOS), cyclooxygenase, lipoxygenase, and cytochrome P450 (metabolic enzymes) [6-8]. The non-enzymatic source of ROS is the mitochondria, commonly known as power house of the cell. In mitochondria, about 2~3% of electrons escaping the electron transport chain (ETC) at complex I and III, react with the molecular oxygen to produce superoxide anion (O_2•−)[9]. Cells typically maintain ROS balance using their antioxidant system. However, excessive ROS levels can disrupt this balance, leading to oxidative stress and contributing to various health issues such as cancer, metabolic issues, and neurodegenerative disorders [10]. Oxidative stress is a biological process that occurs when the generation and accumulation of ROS in cells and tissues exceed the biological system's capacity to detoxify these chemically reactive molecules [11].

From enzyme-catalyzed reactions, NOX is a known membrane-associated multi-subunit protein [12]. It consists of seven isoforms, including NOX1-5 and DUOX1 and 2 [13], which are categorized by their mechanism of action and tissue distribution [14], as shown in **Table 1**.

Table 1. Distribution of NOX isoforms in different tissues of homo-sapiens.

Sr.no	NOX Isoforms	Accession Number	Tissue Expression	Location
1	NOX1	NC_000023.11	Colon	Xq22.1
2	NOX2	NC_000023.11	Appendix, bone marrow	Xp21.1-p11.4
3	NOX3	NC_000006.12	Adrenal, fat, spleen, testis	6q25.3
4	NOX4	NC_000011.10	Endometrium, kidney	11q14.3
5	NOX5	NC_000015.10	Spleen, testis	15q23
6	DUOX1	NC_000015.10	Skin, thyroid	15q21.1
7	DUOX2	NC_000015.10	Gall bladder, thyroid	15q21.1

NOX isoforms that have been specifically detected on the plasma membrane are NOX1, 2, 4, and 5. Additionally, NOX4 is also detected in other cellular compartments, such as the endoplasmic reticulum, nuclear membrane, and mitochondria. These NOX isoforms are also present in specific subcellular microdomains, such as NOX1 at lipid rafts and caveolin, and at invadopodia both NOX1 and NOX4 are found [15]. The NOX enzyme is found in neutrophils and includes three cytosolic subunits, p67-phox, p47-phox, and p40-phox, as well as G-protein Rac, and two membrane subunits (gp91-phox and p22phox) that form the catalytic core, which utilizes NADPH as an electron donor or transfer electron via FAD and heme to molecular oxygen and generate superoxide anions [O2•-]. When stimuli, such as microbial entry, hypoxia, hyperglycemia, and cellular stress, activate protein kinase C (PKC) it phosphorylates p47-phox, and conformational changes expose the SH3 domain of p47-phox. These cytosolic subunits translocate to membrane subunits, and the SH3 domain of p47-phox interacts with the proline-rich region of p22-phox, Rac translocate to the complex independently to activate NOX and assemble the complex. The NOX isoforms (NOX 1, 2, 3, 4, 5 and DUOX 1 and 2) have been observed to have the main gp91-phox subunit. But their mechanisms of activation have changed [16, 17]. To create an active NOX complex, NOX1-3 requires the binding of a membrane protein (p22phox) and cytosolic protein (p67phox, p47phox, and p40phox (only for NOX2)) or the G-protein Rac. The difference lies in the activation of NOX1 and 3 as compared to NOX2. The activation of NOX1 requires interaction with NOXO1 (similar to p47phox), and NOXA1 (similar to p67phox) along with other proteins like tyrosine kinase substrate 4 (TKS4) and Rac1. NOX4 does not need cytosolic protein, only interacts with p22phox, and remains consistently active although activation might occur through an unknown mechanism. Although NOX4 do not need cytosolic subunits for their activation, but they interact with protein DNA polymerase-δ-interacting protein 2 (POLDIP2), and tyrosine kinase substrate 5 (TKS5).On the other hand, DUOX1, DUOX2, and NOX5 activation depends on calcium ions, as they have EF-hands (calcium binding regions)[18, 19]. As shown in **figure 1**.

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Figure 1.

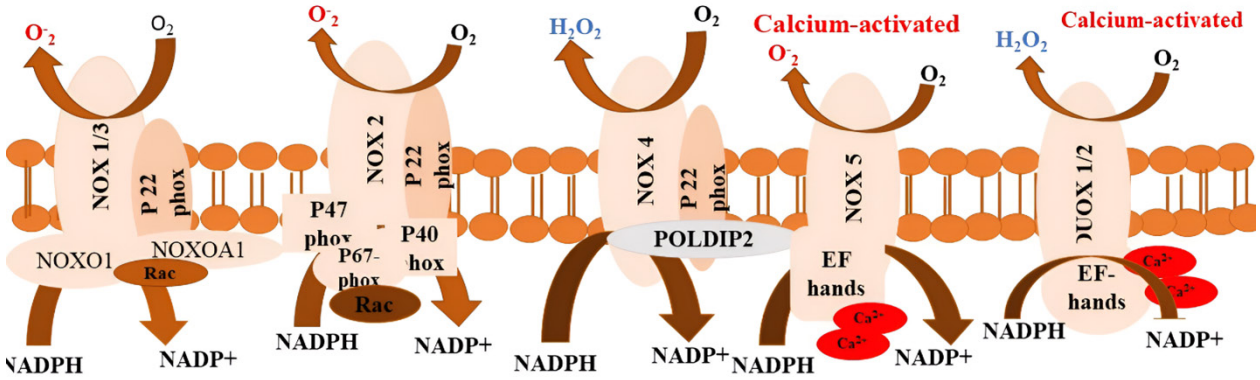


Figure 1. NOX isoforms structures: The NADPH oxidases (NOX) are transmembrane proteins. It consists of seven isoforms named NOX1, 2, 3, 4, 5 and DUOX 1 and 2. These NOX isoforms are classified based on tissue distribution and mechanism of action. The NOX enzymes consist of three cytosolic subunits, p67-phox, p47-phox, and p40-phox, as well as G-protein Rac, and two membrane subunits (gp91-phox and p22phox) that form the catalytic core which uses NADPH as an electron donor or transfers electron to molecular oxygen and generate superoxide anions [O_2•]. The NOX 1, 2, 3, and 5 generate superoxide anions [O_2•-], and the NOX 4 and DUOX 1, and 2 produce hydrogen peroxide (H_2O_2).

The upregulation of NOX and high ROS production by it are seen in cancers [20]. Inside a living body, an appropriate amount of ROS can play a dual role in sustaining the body's normal physiological function, and high levels lead to damaging mitochondrial DNA (mtDNA), inhibition of tumor suppressor genes (TSGs), and proto-oncogenes' activation, which are major etiologies of breast cancer [21]. In breast cancer (BC), uncontrollable growth of abnormal breast cells occurs and forms a tumor, which can spread throughout the body if left unchecked and become fatal. BC originates in the milk-producing lobules and milk ducts. Initially, it is not lethal but can spread to adjacent breast tissue and cause lumps. About 2.3 million women were diagnosed with BC and 685,000 deaths were reported worldwide in 2020 [22]. Among Asian countries, Pakistan has the highest prevalence rate of BC, i.e., one in every nine women has a risk of being diagnosed with BC. Because of a lack of knowledge and proper guidance, Pakistani women are diagnosed with breast cancer at a late stage with a low chance of survival. Early detection is a key to curing BC [23]. In Pakistan, about 90,000 new cases of BC are diagnosed yearly, of which, due to late detection, 40,000 patients lose their lives. According to a recent study, survival rates were 90% if diagnosed early, and 77% of invasive BC occurred in women over 50 years of age [22]. The purpose of this review is to analyze the existing knowledge of the functional role and expression pattern of NOX isoforms (NADPH oxidases) in BC. This review aims to provide valuable insights for researchers and clinicians working towards improved therapies and management strategies for BC treatment and to improve patient outcomes.

THE ROLE OF ANTI-OXIDANT DEFENSE MECHANISMS IN REGULATING OXIDATIVE STRESS

Superoxide dismutase (SOD) is a component of the anti-oxidant system that breaks the superoxide anion [O_2•−] into harmless substances such as H_2O_2. In mammals, three different types of SODs have been identified, each with unique characteristics and specific location in the body. SOD1 (CuZn-SOD) is found in the cytoplasm and has a homodimer structure. It contains two identical subunits that are bound together, both containing copper and zinc atoms. SOD2 (MnSOD) is present in mitochondria and has a tetrameric structure containing manganese. The third type of SOD3 recognized in extracellular space also forms a tetrameric structure containing copper and zinc. Thus, among these three SODs, SOD1, SOD2, and SOD3, each performs a particular function of converting [O_2•−] to H_2O_2 in their respective locations as described [24].

The superoxide radical [O_2•−] is converted to hydrogen peroxide (H_2O_2) with the aid of SOD. Then H_2O_2 is further converted to water by GPX enzymes. SOD prevents •OH production by converting [O_2•−] to H_2O_2 and acts as an anti-carcinogen and anti-proliferative agent. Both [O_2•−] and H_2O_2 play a crucial role in cellular signaling and react to form the hydroxyl radical [•OH] via the Fenton reaction ($Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + \bullet OH + OH^-$). The [•OH] is highly reactive and has a damaging effect on DNA and lipids. The enzymes catalase, GPX (glutathione peroxidase), and PRXs (peroxiredoxin) convert H_2O_2 into water [25], as shown in **Figure 2**.

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Figure 2.

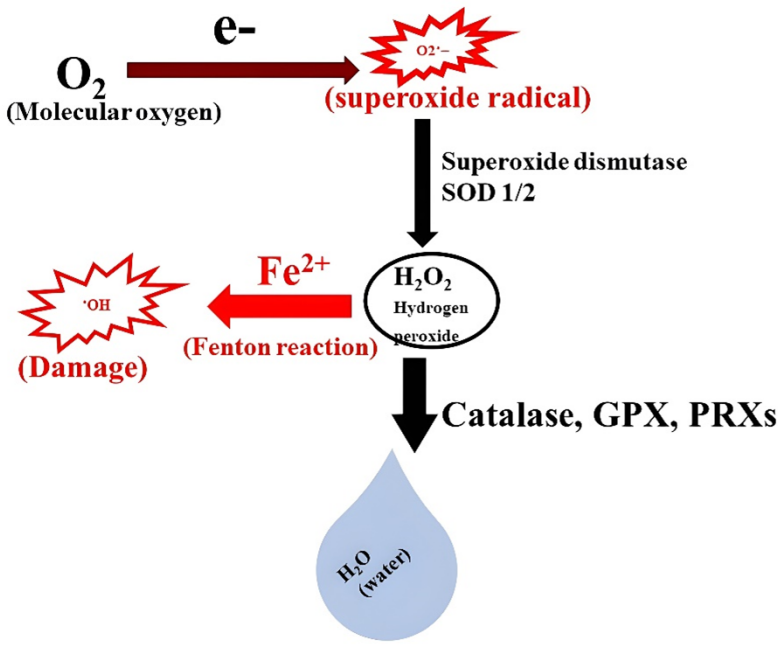


Figure 2. ROS generation and regulation: In mitochondria, the superoxide radical [O_2•−] is produced from molecular oxygen (O_2), which is extracted from nutrients during oxidative metabolism. The superoxide dismutase (SOD) enzymes present in the mitochondria are responsible for the conversion of superoxide molecules into H_2O_2 (hydrogen peroxide). The H_2O_2 is further neutralized and converted into water by the action of the catalyst. Alternatively, the Fenton reaction converts H_2O_2 into a hydroxyl radical [•OH], which is highly reactive and causes damage to biomolecules such as DNA and lipids.

To prevent oxidative stress caused by environmental mutagens and to neutralize ROS, both glutathione peroxidase (GPX) and catalase (CAT) are necessary. Antioxidant vitamins, including vitamins E, C, and A, improve immunity, and inhibit the metabolic activation of carcinogens [25-30]. Pyrosequencing analysis exposed elevated methylation in the promoter region of SOD3 and reduced expression in BC samples compared to normal tissue samples [31]. The low expression of SOD2 was also observed in BC. This reduction in expression is linked to the stage of cancer (TNM system), and nearby lymph node metastasis. The high level of SOD2 in BC is associated with the expression level of estrogen and androgen receptors and the spread of cancer to nearby lymph nodes [32].

H_2O_2 serves as a second messenger due to its uncharged nature, relative stability, and ability to diffuse across membranes [33]. Furthermore, H_2O_2 is recognized as a second messenger in several growth-factor-induced signaling pathways. It regulates the activation of transcription factors including cAMP response element-binding protein (CREB), activating protein-1 (AP-1), nuclear factor erythroid 2-related factor 2 (Nrf2), p53, nuclear factor kappa B (NF-KB), and hypoxia-inducible factor 1 a (HIF-1a). Additionally, H_2O_2 plays a role in epithelial-mesenchymal transition (EMT) [34].

Under oxidative stress condition such as when H_2O_2 is present, it react with cysteine residues to oxidize KEAP1 protein, which disrupts its ability to bind with Nrf2 [35]. Kelch-like ECH-associated protein 1 (KEAP1) is a highly redox-sensitive protein and a member of the BTB-Kelch family. When KEAP1 binds with the cul3 protein, it forms a complex known as a cullin-RINGE3 ligase. This complex plays an important role in protein degradation. One of the main targets of this degradation process is nuclear factor erythroid 2-related factor 2 (Nrf2), which is the key regulator of anti-oxidant systems and is encoded by the nuclear factor erythroid 2 like 2 (NFE2L2) gene [36]. Thus, KEAP1 regulates oxidative stress by controlling the Nrf2 level. In normal conditions, Nrf2 is targeted for ubiquitination and marked for degradation by the 26S proteasome. Oxidative stress induces modifications by oxidizing cysteine residues in KEAP1 and hinders its ability to ubiquitinate Nrf2 and translocate into the nucleus, where it binds to antioxidant response elements (ARE), leading to the activation of transcription of genes encoding the components of an anti-oxidant system [37, 38] [35] [39]. The information has been summarized in **figure 3**.

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Figure 3.

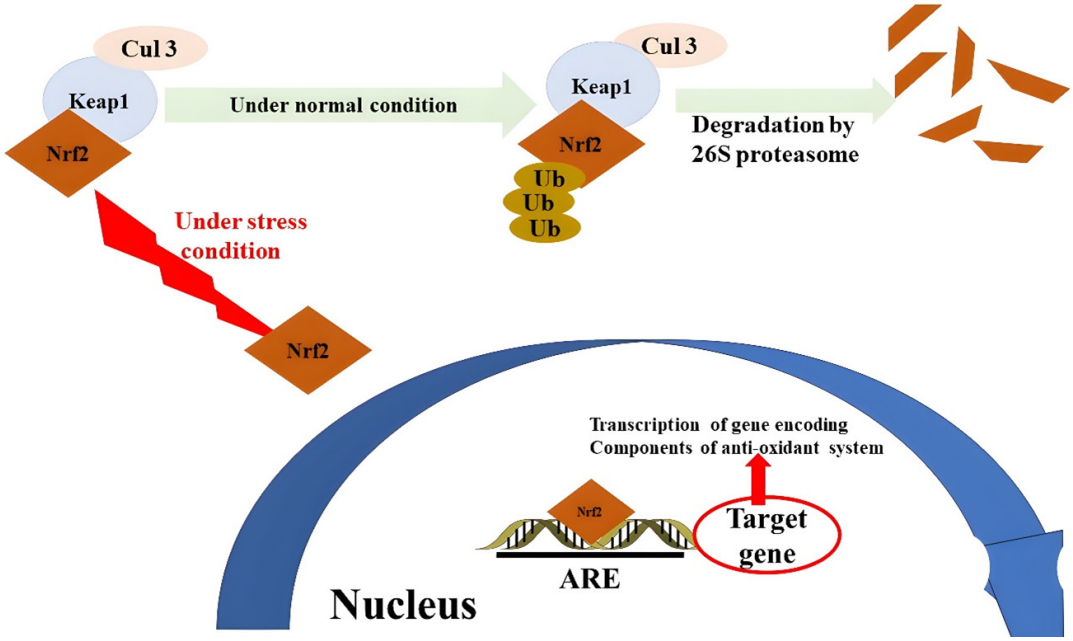


Figure 3. Keap1-Nrf2 pathway: The Keap1-Nrf2 pathway is a cellular defense mechanism regulating oxidative stress. Keap1 binds with Cul3 to form a complex. Under normal conditions, this complex targets Nrf2 for ubiquitination and is degraded by proteasomes. While the oxidative stress condition modifies Keap1, Nrf2 translocate into the nucleus and activates the transcription of anti-oxidant genes by binding to the antioxidant response elements (ARE). The dysregulation of this pathway is implicated in many diseases, such as cancer.

Based on the above discussion, three distinct types of SODs have been identified in mammals each possessing specific characteristics and distribution pattern. Superoxide dismutases (SODs) play a vital role in neutralizing reactive oxygen species (ROS), and maintaining cellular integrity. To prevent oxidative stress GPX and catalase are also necessary. However, under oxidative stress, the Nrf2-KEAP1 pathway emerges as a critical regulator of cellular defense mechanism. Specifically, H_2O_2 acts as a second messenger, oxidizing the KEAP1 protein and inducing conformational changes in its structure. This disrupts KEAP1 binding ability with Nrf2, allowing its nuclear translocation. Then, Nrf2 induced transcriptional activation of antioxidant genes.

NOX ISOFORMS' EXPRESSION IN BREAST CANCER (BC)

The pathophysiological role of NOX in the development of different cancers has been extensively explored. For example, it has been reported by previous studies that NOX1-4 and DUOX2 lead to the activation of angiogenesis and invasiveness via ERK-dependent overexpression of TGF-β1 (tumor growth factor B1), MMPs (matrix metalloproteinases), VEGF (vascular endothelial growth factor), TP53, NF-κB, and HIF-1α [40, 41]. NOX isoforms 2 and 4 are highly expressed in BC cells [42-46].The expression levels and functional roles of each NOX isoforms in BC development are summarized below:

Within the NOX family, NOX2 is a prominent member which shows overexpression and associated with the development of prostate, colorectal, gastric, breast cancer, and myelomonocytic leukemia. The silencing of NOX2 in breast cancer cells leads to a decrease in the expression of inhibitor of NF-κB kinase (IKKε), a key player in cancer progression and chemoresistance [47]. Additionally, another NOX isoform NOX4 interacts with membrane subunit P22phox without the assistance of other cytosolic subunits as compared to other members of the NADPH enzymatic activity, which results in increased production of ROS. NOX 4 is known to be overexpressed in BC patients [46, 48-51] and has proven to promote cancer progression [49]. Notably, NOX4 promotes distant metastasis by elevating lymphatic vessel formation. Additionally, NOX4 stimulates epithelial-mesenchymal transition (EMT) by interacting with tumor growth factor beta (TGF-B), and disrupts glycolysis via the ROS-triggered yes-associated protein (YAP) pathway's activation, thus these events facilitates migration and multiplication of BC cells, ultimately contributing to cancer progression[52-54]. The research was conducted using a method called *in situ* RNA hybridization to analyze whether NOX4 was actively expressed in human BC samples. Their findings show that NOX4 is known for ROS production, causing oxidative stress, and has a role in supporting the stroma of BC. Moreover, researchers found

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that upregulated NOX4 promotes a cellular process called autophagy (in which cells degrade or recycle their components for their survival) in cancer-associated fibroblast cells (CAFs). So, NOX4 supports CAFs survival. Additionally, the researchers found that upregulated NOX4 promotes CAFs survival by activating the protein nuclear factor erythroid 2-related factor 2 (Nrf2), i.e., the oxidative stress regulator, which influenced another gene, Birc5 (baculoviral IAP repeat containing 5). Birc5 promotes cell proliferation by inhibiting apoptosis [55], and appears to play a role in cancer progression. In conclusion, these findings suggest that interaction between NOX4, Nrf2, and Birc5 encourages BC metastasis [56].

Another study indicates that activation of NOX4 increases its activity as a lymphangiogenic factor and may intensify tumor lymphogenesis through activation of the ROS/ERK/CCL21 pathway. In this pathway, chemokine C-C motif ligand 21 (CCL21) is a signaling molecule that acts as a chemotactic factor. The BC cells expressing the CCR7 receptor on their cell membrane can respond to the chemotactic signals of CCL21, which recruit them to lymphatic vessels and spread to remote organs, making them a possible target of anti-metastatic therapy [57]. Cardiac disorder (vascular dysfunction) is a major etiology of mortality in BC survivors, and chemotherapy promotes this menace. They analyzed arteries from women with no record of neoadjuvant chemotherapy treatment (NACT) (doxorubicin, docetaxel, and cyclophosphamide) and from post-menopausal women who had experienced BC treatment with NACT. Thus, no change was found in the expression of NOX 1 and 5, and the overexpression of NOX 2 and 4 was observed in the arteries of women who had experienced NACT [58].

Another NOX isoform, NOX5 may play a dual role in cancer cells as they promote tumor growth through cAMP-response element binding protein (CREB) and Signal transducer and activator of transcription 5 (STAT5A), and encourage cell death by Ca^{2+} and c-ABL. So, NOX5 may regulate the balance between cell death and multiplication in breast, lung, and skin cancer cells [59]. In Malignancies such as breast, lung, hepatocellular, and follicular thyroid carcinoma have low DUOX1 (NOX isoform) expression, while others, including cervical and gastric carcinoma, and pancreatic ductal adenocarcinoma cells, have high DUOX1 expression [60]. It is further validated that the downregulation of DUOX1 in BC cells (MCF12A) accompanies enhanced cell multiplication and reduced adhesion and migration. DUOXA1, which is essential for DUOX1 activation, is overexpressed and affects cell-cell adhesion. DUOX1 might influence the secretion of IL-6 and IL-8 cytokines in endothelial cells during DNA-damaging stress. To test this hypothesis, scientists used the doxorubicin drug to induce stress. The high production of hydrogen peroxide (H2O2) was observed in control cells after induced stress as compared to DUOX1-silenced cells. Thus, DUOX1 regulates cytokine production during DNA-damaging stress [61].

Thus, NOX enzymes are involved in ROS generation, and the upregulation of NOX isoforms leads to increased ROS production, which can accumulate and damage macromolecular structures such as DNA. This enhanced ROS production driven by NOX enzymes, sets the stage for downstream effects on cancer progression such as metastasis as it affects the epithelial-mesenchymal transition (EMT), epigenetic alterations, and drug resistance. So, gaining insights into the NOX enzymes and their involvement in cancer progression via ROS can help us to develop therapeutic strategies.

NOX-DRIVEN ROS: A KEY PLAYER IN THE BREAST CANCER (BC) PROGRESSION

The ROS Impact on the Initiation and Metastasis in Breast Cancer (BC)

Oxidative stress occurs due to high ROS generation, and inadequate defense mechanisms, i.e., anti-oxidant defenses, that counterbalance these molecules are found to play a crucial role in BC [62]. Oxidative stress is a prominent factor, even in familial BC. Molecular biology research has shown that ROS are responsible for epigenetic mutations (an aberrant DNA methylation and histone modifications that result in chromatin remodeling and abnormal gene expression) [63, 64], and interact with biomolecules (DNA, lipids, and proteins). The most common DNA damage caused by ROS is 8-oxo-2-deoxyguanosine (8-oxo-7,8-dihydro-2'-deoxyguanosine,8-oxo- dG) favor substitution mutation (G to T)[65]. Additionally, lipid peroxidation products such as malondialdehyde that interacts with DNA can promote mutation. For instance, malondialdehyde can cause adducts formation with DNA bases (M1G, also known as pyrimido [1,2α] purin-10(3H)-one) and covalent inter strand crosslinks, which means two strands of DNA molecules are joined together by a covalent bond. Both of these variations induce structural changes, thereby inhibiting transcription and replication processes [66, 67].Thus, unrepaired DNA damage can give rise to mutations, resulting in genomic instability, and elevating the risk of potential diseases such as cancer. The study found that the DNA in different parts (stroma, myoepithelium, and epithelium) of the breast is changed by an attack of hydroxyl radical [•OH] on the purine ring. This causes the formation of oxidatively-induced DNA lesions known as 8-OH-dA and 8-OH-dG, which are associated with a higher incidence of BC. The increased frequency of these mutations significantly elevates the risk of BC. The study also observed an uneven distribution of mutations; with older women (ages 50-62) having lower levels of mutations

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as compared to younger women (ages 17-30). This variation in mutation level in different groups of women may be due to the impact of dietary choices and exposure to chemical toxins. It is noticed that, with the increase in women's age, alterations in DNA occur that could increase the chance of BC. The normal gene expression pattern may be disrupted by the structural changes in chromatin, and thus enhances the risk of BC [68].

ROS also play an important role in altering cellular processes and cancer progression through different mechanisms [69]. First, ROS supports cancer growth by immune cells reprogramming such as M2 polarization, T-cell proliferation and differentiation, and enhances the migration of endothelial cells (EC) [69]. High levels of ROS are also linked to aggressive forms of cancer and a stronger immune response. ROS stimulates the immune response against cancer by increasing the activity of immune cells. In the case of BC, elevated ROS is known to enhance the activity of both pro-cancer immune cells (M2 macrophages, regulatory T and T helper type 2 cells) and anti-cancer immune cells (M1 macrophages, dendritic cells, and T helper type 1 cells). Overall, these findings suggest that ER-positive and HER-2 (human epidermal growth factor receptor 2) negative BC patients may have a lower chance of survival because BC cells are under oxidative stress. This oxidative stress correlates with enhanced immune response, and increased cancer aggressiveness [70]. In second mechanism ROS induce metabolic changes by altering glycolytic pathway and mitochondrial dysfunction [69]. Mitochondrial ROS can also lead to oxidative damage to mitochondrial DNA, membrane, and proteins [71]. The inner mitochondrial membrane, where ROS are produced, is vulnerable to lipid peroxidation. This damage alters membrane properties, increasing proton leakage, and disrupting enzymes functions and transporters. Thus, mitochondrial energy production is compromised [72]. ROS affects many metabolic pathways including kreb cycle, glycolysis, fatty acid synthesis, ATP generation [73]. To meet their energy demands, most cancer cells rely on glycolysis [74]. Mitochondrial ROS (mitoROS) increase HIF-1a stabilization and cell multiplication. Hypoxia stabilizes HIF-1a, promoting a metabolic shift towards anerobic glycolysis and reduce oxidative phosphorylation [75] by upregulating glycolytic enzymes and glucose transporters (GLUT), while inhibit PDC (Pyruvate dehydrogenase complex) via pyruvate dehydrogenase kinase 1 activation. This reduced activity of PDC causes decreased ETC flux and kreb's cycle, which may in turn diminish mitoROS generation in hypoxic condition. Also, HIF-1a encourages mitophagy consequences in lessen mitochondrial mass, O2 consumption and resultant ROS generation [76] [69]. The cellular process of EMT is also triggered by hypoxia in many cancers, such as breast, prostate, and oral cancers, as shown by previous studies [77]. Another study reported that in BC, a low oxygen condition called hypoxia enhances levels of hypoxia-inducible factor-1 (HIF-1a). In response to low oxygen, the MRPL52 protein is overexpressed in BC cells. The MRPL52 elevates the survival of BC cells by inhibiting apoptosis and persuading migration to surrounding tissues. The findings of this study suggest that MRPL52 works in two ways: first, it enhances phosphatase and TENsin homolog (PTEN)-induced putative kinase 1 (PINK 1) / parkin-dependent mitophagy to remove damaged mitochondria and prevent increased production of ROS. The second way is the activation of the ROS-Notch1-Snail signaling pathway. Through these two ways, MRPL52 induced BC metastasis and inhibited apoptosis. Thus, MRPL52 can be a potential biomarker and target for BC [78].

In certain cell types, EMT is facilitated by ROS [79]. In EMT (epithelial-mesenchymal transition), cells attain mesenchymal properties by losing epithelial cell characteristics, which are not only involved in wound healing and development but also have the property of metastasis [80]. Epidermal growth factor (EGF) has been examined for inducing EMT in BC cells (MCF-7). EGF stimulates the signaling pathway through the EGF receptor (EGFR), which is overexpressed in BC cells and has a higher chance of cancer progression. A previous study suggested that metformin can suppress the action of EGF by inhibiting the P13K/AKT pathway, which is activated by ROS. The activation of Rac1 G-protein (a component of NADPH oxidases) leads to ROS generation. This causes the oxidation of EGFR and phosphatase enzymes, elevates EGFR-EGF signaling, and promotes metastasis [81, 82]. In BC cell lines (MCF-7 and MDA-MB-231), EGF leads to SMAD 2 and 3 activation, resulting in EMT by decreasing levels of E-cadherin and vimentin[83]. EMT can also be initiated by transforming growth factor beta (TGF-B), which has been recognized as a driver of tumor progression. When TGF-B binds to its receptor, it induces the activation of TFs such as SMAD 2 and 3. These activated proteins, SMAD 2 and 3, form a complex with SMAD 4 in the cytoplasm. Subsequently, these complexes are translocated to the nucleus, where they initiate the transcription of various genes responsible for EMT. Overall, these findings provided insights into a novel therapeutic target for metastatic BC [84]. ERBB2 (ERB-B2 receptor tyrosine kinase 2) is a gene that is overexpressed in BC and considered responsible for BC progression by controlling the expression of two other proteins, including thioredoxin reductase 1 (TXNRD1) and thioredoxin interacting protein (TXNIP). In BC patients, ERBB2 can cause increased expression of TXNRD1, i.e., responsible for the provision of protection against oxidative stress, and decreased expression of thioredoxin interacting protein (TXNIP), which is identified as TSGs. This imbalance in expression between these two, TXNRD1 and TXNIP, is associated with the poor prognosis of BC patients [85-88]. A

study suggested that a small molecule, miR373, is responsible for BC progression by inducing EMT, including various signaling molecules such as TXNIP, ROS, HIF-1a, and TWIST, which together form a signaling cascade. The activation of this cascade might be a potential target for breast metastasis. Intracellular ROS is reduced by either TXNIP silencing or up-regulation of miR-373. When the ROS level is reduced, the BC cells behave more aggressively, which leads to carcinogenesis by activating HIF1a and TWIST pathways. Thus, activation of the miR-373-TXNIP-ROS-HIF-1a-TWIST signaling cascade is related to poor outcomes in BC patients [89].

A longitudinal study comprised 60 women, equally divided into a control group (n = 30), and a patient group (n = 30). Both groups were assessed for their oxidative stress biomarkers (peroxynitrite, gamma-glutamyl transferase, and malondialdehyde (MDA) and serum levels of anti-oxidant biomarkers (ceruloplasmin, glutathione, and ascorbic acid). In BC patients, low levels of glutathione and ceruloplasmin were found. Both of these are beneficial substances. While vitamin C levels don't seem to be linked to BC, additionally, high levels of MDA and peroxynitrate were observed as harmful molecules due to their adverse effects on BC. The dysregulated levels of these molecules are used as biomarkers for early detection of BC [90].

Previous research has shown the increased activity of NADPH oxidases (NOX1, 4, and 5) in BC. It is suggested that NOX 4 may produce H2O2 in mitochondria, which helps cancer cells resist cell death caused by the chemotherapeutic drug (etoposide). Moreover, NOX4 encourages cell invasion and EMT and promotes tumor progression by inhibiting apoptosis. The mechanism involves the molecule tumor growth factor beta (TGF-B), which phosphorylates SMAD2 and 3, and activates SMAD and (Jun N-terminal kinase) JNK (Jun N-terminal kinase) activation. This leads to increased NOX4 production. Alternatively, AKT1 i.e., another signaling molecule, also elevates the NOX4 generation which in turn causes cellular changes such as F-actin polymerization, invapodia formation, and cancer progression. In addition to NOX4, another enzyme, SOD2 i.e., is involved in carcinogenesis at late stage III. It achieves this by influencing AMPK (AMP-activated protein kinase) phosphorylation at Thr 172. It directs the cancer cells to a metabolic process of glycolysis from cellular oxidative respiration. Ultimately, it contributes to cancer progression by improving cell survival and preventing programmed cell death [54, 91]. NOX 4 also mediated mtROS signaling increasing the stability of mRNA coding for MMP9. Thus, this pathway may facilitate cancer cells to spread to nearby tissues but may not have an impact on the growth of the tumor[92]. When researchers treated BC MCF-7 cells with a substance called phorbol ester (TPA) which activates protein kinase C (PKC), the activated PKC leads to encouraging cell motility and growth rate. The increased growth and movement of cancer cells (EMT) was accompanied by increased expression of slug. The increased slug expression by TPA was regulated in two ways. The first was the promotion of acetylation of histone H3 protein and the second was the NOX2 signal that leads to ROS production. Overall, ROS generation and histone acetylation play a role in EMT and cancer progression [93]. Malic enzyme1 (ME1) converts malate to pyruvate and NADP+ to NADPH. The elevated expression of ME1 in breast cancer is associated with large- sized tumors. In this study, researchers analyzed ME1 expression in two types of breast cancer cells. In MCF-7, the increased levels of ME1 were examined to enhance mobility and EMT but reduced ROS. Moreover, decreased levels of ME1 in MDA-MB-468 produced results contrary to the results observed in case of MCF-7. So, ME1 can be a drug target and biomarker for BC metastasis [94].

As oxidative stress observed to play a vital role in BC development, because BC patients often show mtDNA (mitochondrial DNA) changes. Mitochondria are known as the powerhouse of the cell, which is responsible for energy production. Mitochondria contain their DNA, called mtDNA. The mtDNA lacks genes for histone and DNA repair proteins, which are present in the nuclear DNA (gDNA). Because the mitochondrion is the primary site of cellular respiration and energy production, it is constantly exposed to ROS as a by-product during these processes. Due to constant exposure and limited capacity to repair, mtDNA experiences a higher mutation rate than gDNA. As a compensatory mechanism, mitochondria increase the mtDNA copy number in response to damage, in contrast with gDNA, whose integrity is maintained by the repair system. The increased mtDNA copy number can provide insight into DNA damage and oxidative stress [95, 96]. A study shows that by analyzing gene-expression data from the TCGA (the cancer genome atlas) tumor dataset, researchers observed changes in the mitochondrial structure between BC and non-cancerous cell lines as well as function among different subtypes of BC. Moreover, in triple-negative BC (TNBC) cell lines, a high level of ROS was observed. Thus, findings indicate that TNBC cells need class of substances for survival called ROS, which, when treated with antioxidants, results in cell death. So, mitochondria are the main source of ROS in TNBC. Apart from this, estrogen receptor-positive BC ER+ cells were exposed to the same antioxidant treatment, but cells were able to survive [97]. It might be that ER+BC has an alternative mechanism for survival that is not dependent on ROS. A case study shows that the D5 haplogroup, i.e., a specific genetic lineage of human mtDNA, is particularly associated with the development of BC. The process begins with the activation of the AKT (AKT serine/threonine kinase) protein, which regulates cell growth and survival. In D5 cybrids (cells with mtDNA from the D5 haplogroup), there is

a high level of ROS, which leads to activation of the AKT protein through phosphorylation of a specific site (Thr 308) on the AKT protein. This study demonstrated that there is a decreased mitochondrial function in cells with the D5 haplogroup in contrast with cells with non-D5 haplogroups. Thus, D5 haplogroup cells have a high level of ROS and thus lead to activation of AKT, which in turn results in tumor progression. These factors contribute to the growth of cancer cells in breast tissue linked to the D5 haplogroup [98]. Another group of researchers carried out a genome-wide analysis of mtDNA extracted from BC patients to investigate the germline mutation. Scientists have found that germline mutations can promote a higher risk of getting BC, especially if the individuals already have a family history of the disease. Scientists analyzed mtDNA obtained from 83 BC patient samples and 22 from healthy individuals. About 383 mutations were detected in BC patients and 70 mutations in healthy individuals. Notably, 283 mutations (232 in patients and 88 in healthy individuals) had previously been recorded. Researchers analyzed 32 different mutations in mtDNA that significantly vary between normal individuals and BC patients. To analyze the risk associated with these mutations, researchers found 27 out of these 32 mutations linked to a decrease in the risk of BC. On the other hand, 5 mutations (C6296T site mutation, A8860G site mutation at ATP6, deletion in the 2463A site of RNR2, 13237A site deletion at ND5, and 6298T site deletion at COX1) were associated with high-risk BC and were suggested to be novel indicators of BC that may have future therapeutic applications. The mtDNA codes for components of redox enzymes. So, mutations in the mtDNA lead to dysfunctional proteins, which affect cellular processes and ultimately lead to a physiological disorder such as cancer [99].

Many drugs are used to treat BC by targeting oxidative stress by reducing ROS levels. In contrast, increasing ROS levels with the aid of therapeutics to induce apoptosis, causing unrepairable DNA damage, and mitochondrial dysfunction, all contribute to cancer cell death [21], as shown in T**ables 2 and 3**.

Table 2. ROS-lowering drugs used for breast cancer management.

Sr. No	Drugs	Nature	Biological Role	Reference	Pubchem ID
1	Elemene	terpenes	Scavenging ROS and preventing metastasis	[100]	6918391
2	PD (polydatin) combined with +2-DG		Reduced ROS and inhibition of P13K/AKT pathway	[101]	30920152
3	Hyperoside	flavonoid	NF-KB signaling	[102]	5281643
4	Vitamin E with doxcorobicin	antioxidant	Effect of doxcorobicin increases with vitamin E and high dose leads to cytotoxicity of doxcorobicin	[103]	-
5	Vitamin C	antioxidant	Prevents breast cancer	[104]	54670067

Table 3. Known drugs that treat breast cancer by increasing ROS.

Sr.No	Drugs	Nature	Biological Role	Status	Drug Id	References
1	*Combination Of Nic And Doc	Antihelminthic, Anticancer	Activation Of Wnt Signaling And Cell Cycle Arrest (G0&G1), Increases Ros	Both Approved	4477 31703	[105]
2	Fri-1	Anti-Cancer Isoinolinequinone	Dysregulation Of Mitochondrial Bioenergetics	-		[106]
3	*A-M	Anti-Tumor, Microbial And Anti-Oxidant	Induces Apoptosis By Causing Mitochondrial Dysfunction	Not Approved	5281650	[107]
4	Epicatechin	Anti-Cancer	Induction Of Apoptosis	Investigational	72276	[108]
5	Crocin	Anti-Cancer		Investigational	5281233	[109]
6	Chrysophanol	Anti-Tumor And Anti-Angiogenetic, Anti-Inflammatory		-	-	[110]
7	*Zerumbone	Phytochemical		-	5470187	[111]
8	Combination Of Thymoquinone And Lebanese Propolis	Anti-Oxidant And Anti-Cancer	Induction Of Oxidative Stress	Investigational	10281	[112]

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9	Costunolide	Anti-Cancer	Induction Of Apoptosis	-	5281437	[113]
10	Protodioscin And Dioscin	Natural Steroidal Compounds	Altered Enzymatic Activities Of Glutathione Reductase And Thioredoxin Reductase.	-	119245 441891	[114]

*Nic= Niclosamide, Doc= Doxorubicin, α-M= α-Mangostin, * zerumbone induced oxidative stress and resensitize BC cells to paclitaxel.

In summary, oxidative stress results from an ROS-antioxidant imbalance. As individual's age, their antioxidant capacity decreases. ROS-driven damage to genomic DNA and mtDNA contributes to breast cancer initiation and metastasis. The mtROS production in TNBC is significantly higher as compare to other subtypes of BC. ROS also mediate BC progression via multiple mechanisms including epithelial mesenchymal transition (EMT) by tumor growth factor beta (TGF-B), and by MRPL52 protein overexpression in response to hypoxia. TNBC lacks targeted therapy thus targeting ROS offers a promising therapeutic target.

Role of ROS in the Induction of Drug Resistance in Breast Cancer (BC)

The conventional cancer therapies are chemotherapy and radiotherapy. These therapies elevate ROS levels in cancer cells to induce cell death. But simultaneously, many antioxidant pathways are activated in cancer cells, which inhibit ROS generation induced by chemotherapy and, radiotherapy thus such cells eventually leading to drug resistance [115]. The ATP-binding cassette (ABC) transporters family comprises 48 members in humans. These ATP transporters facilitate the transport of substances across the cell membrane by using energy derived from ATP hydrolysis. Several members of this family, including multidrug resistance-associated protein 1 (MRP1), p-glycoprotein (P-gp), and BC resistance protein (BCRP), are highly expressed in drug-resistant cancer cells. These receptors contribute to drug resistance by expelling drugs out of the cell. One of the ABC transporters, BCRP, encoded by the ABCG2 gene, was thought to be localized in cell membranes only. However, recent research indicates its location also in some cellular compartments, including the nucleus, cytoplasm, and mitochondria. The BCRP acts as a drug pump that expels drugs out of the drug-resistant cancer cell, and its high expression is associated with BC and leukemia [116]. In a study, researchers exposed cancer cells to Di (2-ethylhexyl) phthalate (DEHP). This exposure is known to make cancer cells more resistant to chemotherapeutic drugs, and these drugs become less effective for BC patients. Researchers further investigated whether this exposure leads to overexpression of the ABC transporter. This overexpression resulted in more drug export and efflux and decreased drug accumulation inside the cell. Thus, it becomes difficult for chemotherapeutic drugs to work properly. It is therefore required that potential inhibitors be developed to block these transporters and improve drug efficacy during the treatment of BC [117].

Hypoxia increases hypoxia-inducible factor 1 alpha (HIF-1a), a transcription factor that is involved in different aspects of cancer progression, including angiogenesis, immune escape, drug resistance, and metastasis [118]. In BC cells, the ACE2 (angiotensin-converting enzyme 2) gene is examined to depend upon the ROS-AKT-HIF-1a pathway. Through this pathway, the ACE2 gene responds quickly to chemotherapeutic drugs, and expression of the ACE2 gene is increased. When ACE 2 protein levels elevate, it induces the intracellular ROS level, and high levels of ROS enhance AKT phosphorylation, and afterwards increase HIF-1α expression, which in turn elevates ACE2 expression. The highly expressed ACE2 not only affects cancer cells' response to chemotherapy but is also associated with a worse prognosis in BC patients [119]. Under hypoxia, the breast tumor replicating cells (TRCs) can proliferate via metabolic reprogramming. Hypoxia triggers the activation of transcription factors Foxo1 (forkhead box proteinO1) and HIF-1a which promotes epigenetic alterations to increase the expression of phosphoenolpyruvate carboxykinase (PCK1). PCK1 is a key enzyme that catalyzes a series of metabolic reactions. Among these is gluconeogenesis, in which glucose is synthesized from non-carbohydrate precursors. Then glycogenesis, converting glucose to the stored form of glycogen; then glycogenolysis, which is the breakdown of glycogen into glucose; and the pentose phosphate pathway (PPP), which generates NADP as a by-product that facilitates reduced glutathione antioxidant generation. Glutathione slightly increases the ROS level. This rise in ROS levels helps breast TRCs grow under hypoxia [120]. In another study, scientists observed the effects of HIF-2a, in two types of BC cells, MCF-7 and T47D. It was noticed that HIF-2a, when induced by hypoxia, makes BC cells behave like stem cells which have the ability to resist drugs. When HIF-2a was blocked either genetically or via the use of a drug (YQ-0629), it restricted the stem cell-like behavior of cancer cells. These were the outcomes in both in vivo and in vitro analyses. HIF-2a works by inducing the cell to produce a SOD2 enzyme that reduces mtROS. When fewer mtROS are transported from mitochondria to the endoplasmic reticulum (ER), it in turn reduces the activity of protein disulfide isomerase (PDI) in the ER. Usually, PDI competes with another protein, glucose regulating protein (GRP78) (78), which it allows to bind with misfolded proteins and switch off the stress response, which is the unfolded protein response in the endoplasmic

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reticulum (UPRER) pathway. This activation of the stress response leads to cancer progression. If protein disulphide isomerase (PDI) is less active, it allows more binding of GRP78 with misfolded proteins and switches on the UPRER pathway. Thus, by inhibiting PDI, GRP78 works better by activating the stress response system in BC cells [121].

Cancer stem cells (CSCs) play an important role in the failure of conventional therapies. In BC, these CSCs have certain characteristics, as they are either CD24-low or CD44+ and express high levels of the aldehyde dehydrogenase enzyme. The same characteristics, CD24- low/CD44+, are also seen in cells that change from epithelial to mesenchymal in epithelial-mesenchymal transition (EMT). CD24 is a surface marker (protein) that is recognized in many cancer cells but its role in cancer progression and drug resistance is unknown. But contrary to this, some researchers have found that the loss of CD24 makes cancer cells (HMLE BC cells) more resistant to conventional therapies. This resistance is due to low ROS levels, and CD24 seems to control ROS production by affecting the functioning of mitochondria. Thus, CD24 can be a target as it is involved in making BC cells resistant to treatment [122]. Few researchers grew BC cells and BCSCs in the lab and compared miRNA expression patterns between them. A specific type of Micro RNAs (miRNA), has-miR-27a, reduces a patient's survival and induces chemoresistance in BC cells. While lower levels of has-miR-27a produce the same effect in BCSCs like cells grown in the mammosphere. Artificially increasing the has-miR-27a levels resulted in a decrease in mammosphere size. Moreover, elevating the levels of has-miR27a made BCSCs more sensitive to anti-cancer drugs by lowering the activity of genes responsible for protecting cells from ROS. Thus, the use of has-miR-27a can be a potential therapy approach for BC [123].

In BC patients, the major cause of cancer metastasis and failure of conventional therapies is the presence of BC stem cells (BCSCs). These BCSCs exhibit high expression of ABC transporters, i.e., BCRP, MDR1, and MRP8, and tend to spend more time in the G0 phase, i.e., the non-cell dividing phase. The use of both strategies helps them escape from the toxic effects of drugs. These BCSCs are better at repairing DNA damage caused by these therapeutic molecules. The targeting of BCSCs is an approach to overcome therapeutic resistance in any subtype of BC, including TNBC, HER2, luminal A, and B. The complex interaction between BCSCs and stromal cells makes them resistant to hormone therapy (fulvestrant, tamoxifen), chemotherapy drugs (anthracycline, platinum, and paclitaxel), and lapatinib, trastuzumab (HER2-targeted drugs). Furthermore, the rewiring of signaling pathways including Notch, P13K /AKT / mTOR, hedgehog (Hh), and Wnt/β-catenin also contribute to drug resistance in BCSCs [124].

Based on discussion, conventional therapies increase ROS levels to kill cancer cells, but cancer cells activate antioxidant defenses, reducing therapy effectiveness. Some of the ATP transporter family members contribute to drug resistance by expelling drug out of the cell. From these members BCRP (Breast cancer resistance protein) observed to be highly expressed in breast cancer and leukemia. In breast cancer (BC) patient, the main cause of conventional therapy failure is BC stem cells, which exhibit high expression of MDR1 (multi drug resistance mutation 1) and MRP8 (multidrug resistance- related protein) in addition to BCRP. In contrast chemotherapeutic drugs increase the level of ACE2 (angiotensin-converting enzyme) in breast cancer via ROS-AKT-HIF-1α axis. The elevated ACE2 protein increases the intracellular ROS, which phosphorylates AKT and subsequently increasing HIF-1a expression which, in turn upregulates ACE2 expression. The highly expressed ACE2 associated with altered cancer cell's response to chemotherapy and poor outcome in BC patients.

Epigenetic Alterations Induced by ROS in Breast Cancer (BC) Cells

The DNA damage caused by ROS leads to epigenetic and genetic alterations [125]. ROS interacts with DNA and induces epigenetic mutations such as site-specific DNA methylation, histone phosphorylation, and histone acetylation. All these changes can alter the chromatin structure. Surprisingly, some epigenetic changes due to ROS can retard cancer progression by activating tumor suppressor genes (TSGs), including P53, and PTEN, and by repressing MYC, which is an oncogene [126]. Epigenetic modifications do not directly alter the nucleotide sequence of the DNA. However, such variations can affect genetic and cellular processes by modulating the expression of various genes. Eventually, such genomic changes can result in various diseases, among them aging and cancer. A group of researchers reported that the KMT2D gene, a methyl transferase-encoding gene, is mutated in approximately 6% of TNBC and is associated with poor patient's survival [127].

BC is a complex disease influenced by both epigenetic and genetic mutations. The hallmark feature of BC is genomic instability, which leads to mutations and copy number variations. On the other hand, epigenetic modifications affect gene expression patterns by causing variations in the chromatin structure through epigenetic processes such as DNA methylation, histone acetylation, etc. Thus, both of these processes lead to disturbances in cellular processes and BC metastasis. In breast cancer, epigenetic mutations occur frequently, regardless of the initial genetic triggers. The progression of breast cancer via primary genetic mutations is not consistently linked to TSGs and oncogenes; epigenetic changes always follow the genetic instability contributing to the progression of the disease [128].The DNA damage by ROS leads to epigenetic and genetic alterations that diminish TSG

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activity [129]. Both genetic and epigenetic alterations lead to changes in PTEN expression, a TSG that is closely linked with BC metastasis. The PTEN gene is linked with BC progression via many mechanisms, such as mutations, epigenetic silencing, and post-translational modification [130]. In BC, the epigenetic mutations are due to myelocytomatosis oncogene (MYC), i.e., a tumor reprogramming factor in epithelial cells of breast tissue, contributing to the possible progress in treating basal-like breast cancers [131].

In BC MCF-7 cells, researchers identified that the ROS-induced EMT depends on the distal-less homeobox-2 (Dlx-2)/snail signaling pathway. The Dlx-2 gene regulates snail expression, suppressing E-cadherin, i.e., cell-adhesion proteins, thus, promoting EMT [132]. In another study, researchers observed the role of tumor growth factor beta (TGF-B) in EMT. To investigate the mechanism of EMT induced by TGF-B, a detailed analysis of TGF-B-treated cells was carried out. The results indicated several alterations in the nuclear structure, including an oval shape, enlarged size, and hypochromatic chromatin. There was no change in the DNA methylation pattern. However, reduced levels of H3K9Me2 and E-cadherin, as well as increased levels of H3K36Me3 and H3K34Me3, were observed. It was therefore suggested that TGF-B is capable of inducing epigenetic modification in the chromatin, which leads to promoting EMT. Lysine-specific demethylase 1 (LSD1), which is the negative regulator of EMT, is known to inhibit TGF-β signaling and thus result in increased EMT-driven cell motility and the induction of chemotherapeutic resistance in cancer cells [133, 134].

Epigenetic alterations modify gene expression pattern without altering the DNA nucleotide sequence. ROS induced epigenetic modifications by altering oncogene expressions. ROS-mediated oxidative stress alters the key processes of epigenetic which are DNA methylation and histone modification, and leads to chromatin remodeling. Consequently, tumor suppressor genes (TSGs) such as PTEN (phosphatase and tensin homolog gene) are silenced, and activation of oncogenes like MYC. The TSGs can be silenced via DNA methylation in contrast oncogene activated by removing methylation marks. ROS-induced epigenetic alterations trigger cancer initiation, progression, and chemotherapy resistance. Understanding ROS and epigenetic interactions is vital for cancer prevention and therapy.

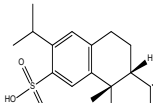
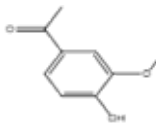
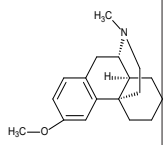
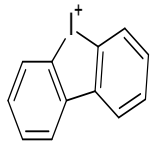
NOX LANDSCAPE AND THEIR INHIBITORS IN BREAST CANCER (BC)

As NOX is associated with cancer development, it is considered the prime target candidate during drug design and development for the treatment of cancer. So far, numerous natural and synthetic organic/inorganic and peptide-based NOX inhibitors have been discovered to treat cancer [135]. A study has reported that inhibition of NOX2 (in breast cancer, IKK over-expression depends on NOX2) using Gentian Violet, Brilliant Green, and Nitroxide Tempol can reduce the expression of an IkappaB kinase ε (IκB) oncogene involved in invasion, transformation, and chemoresistance in MCF-7 and ZR75.1 BC cell lines [136]. Another study has reported that NOX4 (which contributes an oncogenic characteristic localized to mitochondria), mediates lymph angiogenesis and metastasis in BC cells via dysregulating the ROS/ERK/CCL21 pathway. However, this effect of NOX4 has been reduced upon its inhibition using GKT137831 (Setanaxib), 58496428, or shRNA [137]. Moreover, Plumbagin (5-hydroxy-2-methyl-1,4-naphthoquinone) is also used as a NOX4-specific inhibitor and has been proven to have anti-proliferative effects in BC cells [138, 139], inactivate MMP-9, kt/NF-KB, and VEGF pathways, as it is investigated for its anti-cancer activity (DB17048). NOX5 expression significantly increases due to the up-regulation of its transcription factor STAT5A in BC cells [140]. Dihydrotanshinone (DHTS) has been previously employed to treat NOX5-induced BC [141]. Some known NOX inhibitors are discussed in **Table 4**.

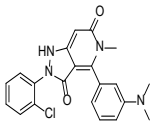
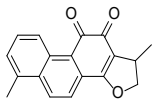
Although seven isoforms of NOX are known as discussed earlier, only inhibitors of some isoforms are used to target several cancers and other diseases. The main focus of our paper is to evaluate the therapeutic role of NOX inhibitors in the treatment of breast cancer (BC). Till date only two NOX inhibitors, setanaxib and dihydrotanshinone, have been proven effective for BC cell lines, but both have not been validated, through clinical trials for therapeutic application for BC patients. Setanaxib targets lymphangiogenesis and dihydrotanshinone and inhibits cancer stem cells formation in BC.

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Table4. Known NOX inhibitors.

Sr. no	NOX isoforms	Drugs	Drug ID	Class of compound	Mechanisms of action	Chemical formula	Targets	Function	Other Therapeutic Application	Cancers	Clinical trials	status
1	NOX 1 1*	Ecabet (also NOX3 inhibitor in the treatment of ulcer)	 (PubChem ID: 65781) DB05265[142]	diterpenoids	(1) Improves mucin production by corneal epithelia and conjunctival goblet cells, and prevents moisture loss by improving tear film lubrication. (2) In the treatment of ulcers reduces H.pylori survival and inhibits pepsin activity.	$C_{20}H_{28}O_5S$	(1) Mucin (2)H.pylori	antagonist	Dry eye syndrome Peptic ulcer	unknown	NCT00370747 NCT01308177 NCT01039558 NCT00667004	investigational
		Apocynin	 (PubChem ID: 2214) DB12618 [143-147]	alkyl-phenylketones.	Inhibit NOX through inhibition of its subunit gp91 (phox) mRNA expression and suppresses the IL-10 and TNF-alpha generation.	$C_9H_{10}O_3$	Inhibit NOXO1 that activates NOX1	Inhibitor	Hypertension, Atherosclerosis, Asthma Chronic Obstructive Pulmonary Disease.	Lung and prostate cancer	NCT03680638 NCT00992667	investigational
2	NOX 2	Dextromethorphan (DXM)2*	 (PubChem ID: 5360696) DB00514	morphinans	reduces pain perception by blocking NMDA receptors, providing analgesic effects.	$C_{18}H_{25}NO$	NMDA receptor antagonist	inhibitors	Dry cough, atherosclerosis, inflammation, neurotoxicity	solid tumors, leukemia, lymphoma, breast cancer2* Hodgkin and non-hodgkin lymphoma	NCT00605605 NCT00728468 NCT00176553 NCT00003687 NCT00873366 NCT06383338	Approved
3	NOX 3	Diphenyleneiodonium (DPI)	 PubChem ID: 3101 DB17025 [148, 149],[150]	iodonium class	(1) inhibiting mitochondrial respiration reduces the oxidative ATP production in the mammosphere of CSCs derived from human BC cell line MCF7 cells. (2) Control inflammatory biomarkers in lung inflammatory diseases.	$C_{12}H_8I$	(1) Target mitochondrial respiration in CSCs. (2) inflammatory biomarkers (oxidative stress and pro-inflammatory cytokines) in lung inflammatory disease.	inhibitor	Lung inflammatory disease	Cancer stem cells (CSCs) in BC Colorectal cancer (CRC)3*	Not reported	investigational

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4	NOX 4	Setanaxib	 (PubChemID: 58496428) DB16869 58496428 GKT13781 [57, 151]	pyrazolo-pyridie dione	reduces lymphangiogenesis and tumor metastasis in BC	$C_{21}H_{19}ClN_4O_2$	Inhibit NOX1- and NOX4- mediated signal transduction pathways	inhibitor	Alport syndrome, liver stiffness,	Breast cancer primary biliary cirrhosis, squamous cell carcinoma of head and neck,	NCT06274489 NCT05014672 NCT05323656 NCT03226067	investigational
5	NOX 5	Dihydrotanshinone (DHTS)	 (PubChem ID: 5316743) 5316743 11425923 [141, 152, 153]	lipophilic abietane diterpenes	(1) In infantile hemangioma (IH) it elevates apoptosis-related proteins (caspase3,8,9 and BAX) and inhibits angiogenesis by reducing MMP9 and VEGFR2. Thus, exhibit anti-apoptotic or anti-angiogenic activity. (2) Inhibit CSCs formation in BC via NOX/ROS/STAT3/IL-6 pathway	$C_{18}H_{14}O_3$	(1) Target angiogenesis and tumorigenesis pathway in IH. (2) target CSCs in BC	inhibitor	Cardiovascular diseases, hepatitis and inflammation	Infantile hemangioma (IH) Breast cancer	Not reported	approved
6	DUOX 1	Not reported	-					-	-			-
7	DUOX 2	Not reported	-					-	-			-

1*NOXO1 encodes an NADPH oxidase (NOX) organizer, positively controlling NOX1 and 3. The protein contains two SH3 domains and PX domain Gene ID: NC_000016.10. Dextromethorphan hydrobromide2* is used in breath tests for women diagnosed with breast cancer (BC) and receiving tamoxifen treatment. Apocynin and DPI3* are used as an anti-inflammatory in the CRC model by reducing macrophagic-mediated inflammatory response.

CONCLUSION

Through an evaluation of current knowledge, this review provides key insights for future perspectives on breast cancer (BC) diagnosis and treatment. This review highlights the key role of ROS in every stage of BC biology (i.e., from initiation to metastasis via EMT). Although ROS are generated through various mechanisms, NADPH-oxidases (NOX) are the major facilitators. Various isoforms of NOX are known, and their up-regulation in BC patients has been validated worldwide. Hence, targeting NOX is suggested to be a better therapeutic approach for BC. It is therefore suggested that NOX over-expression might be better regulated through either silencing the corresponding gene using the RNA interference method, editing the gene via the CRISPR-Cas9 technique, or inhibiting the NOX-related enzyme activity through specific inhibitors. Many NOX inhibitors have been developed by the researchers, but none of them is being used clinically for BC treatment. While other proposed aspects need to be thoroughly investigated. Although many NOX inhibitors are known, their underlying mechanisms have not been completely understood. To improve the efficiency of treatment, combination therapies can be used, such as NOX inhibitors, along with chemotherapy and radiation. Analyzing tumor

microenvironment interaction with NOX will help identify more potential targets. A complete validation of the individual NOX isoforms in cancer development requires further animal modeling-based studies.

Highlights

- NADPH oxidases (NOX) are trans-membrane proteins that exist in seven isoforms (NOX1–5 and DUOXI–2), each of which has a unique location and mode of activity.
- The synthesis of reactive oxygen species (ROS) is significantly aided by NOX.
- ROS normally functions as a signaling molecule in cellular processes like apoptosis and the immunological response.
- ROS related oxidative stress has a role in the onset and metastasis of BC and induces epigenetic changes, and drug resistance in BC cells.
- Many therapeutic drugs are used to relieve oxidative stress but NOX inhibitors are not in common practice.

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