

Review Article

Therapeutic Potential of Targeting Nrf-2-Keap-1 Signaling in Breast Cancers

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Abstract

Nuclear factor erythroid 2-related factor 2 (Nrf-2) is a stress activated transcription factor, which regulates the expression of genes involved in the (a) degradation of reactive oxygen species (ROS); (b) drug metabolism and export; (c) formation of new blood vessels (angiogenesis); and (d) cell proliferation and apoptosis. Kelch-like erythroid cell-derived protein 1 (Keap-1) is the major regulator of Nrf2 activity in cells. In normal cells Keap1 binds to Nrf2 thereby prevents its translocation to nucleus. However, in cancer cells Keap-1, due to mutations or unusually high oxidative stress, has lost its ability to effectively bind and prevent Nrf2 translocation to nucleus. Therefore, deregulated expression and activity of Nrf2 is widely reported in cancers. But, it is not clear whether targeted inhibition or pharmacological activation of Nrf2 is required for effective inhibition of tumor cells growth, as many studies have reported a dual role for Nrf2. While many studies have reported targeted inhibition of Nrf2 inhibits tumor growth, few recent studies have demonstrated that pharmacological activation of Nrf2 helps to prevent the transformation of normal cells in to cancer cells. Moreover, it is not known whether Nrf2 is a good therapeutic target to treat carcinomas of breast. Hence, in this article we address these key questions by reviewing recent developments highlighting the functional role of Nrf2 and its target genes in the development of cancers with primary emphasis on breast cancer, followed by evaluating the potential of pharmacological agents that inhibit tumor growth by modulating Nrf2 activity. Finally, current status of Nrf2 modulators in the clinical development for treating carcinomas of breast and other organs is also addressed.

Keywords

- Nrf2
- Keap-1
- NQO1
- HIF1 α
- HO1
- Reactive oxygen species
- Breast cancer

ABBREVIATIONS

4f2hc: 4F2 Cell-Surface Antigen Heavy Chain; ABCC: ATP Binding Cassette Subfamily C Member; ABCB1: ATP Binding Cassette Subfamily B Member 1; AKT: AKT Serine/Threonine Kinase 1; AMPK: 5' Adenosine Mono Phosphate-Activated Protein Kinase; ARE: Antioxidant Response Element; Bach1: BTB and CNC Homolog 1; BCRP: Breast Cancer Resistance Protein; BRAC1: Breast Cancer Type 1; BTB: Broad Complex, Tram-Track, Bric-A-Brac; Bzip: Basic Leucine Zipper; C: Cysteine; CAI: Carbonic Anhydrase Inhibitor; Cav1: Caveolin 1; CDDP: Cis-Diamminedichloridoplatinum (Cisplatin); Chk1: Checkpoint Kinase 1; CNC: Cap 'N' Collar; CTR: C-Terminal Region; Cul3: Cullin-3; CXCRB: C-X-C Motif Chemokine Receptor; Cyp3a4: Cytochrome P450 Family 3 Subfamily A Member 4; Cys: Cysteine; DDR: DNA Damage Response; DGR: Double Glycine

Repeat; DHP: Dehaloperoxidase; DMBA: 7,12-Dimethylbenz (A) Anthracene; Dnam: DNA Methylation; DPP3: Dipeptidyl Peptidase 3; DSS: Dextran Sulfate Sodium; ER: Estrogen Receptor 1; ER: Estrogen Receptors; ERK: Extracellular Signal-Regulated Kinases; FAAH: Fatty Acid Amide Hydrolase; FOP3: Fork head Box P3; Fyn: Tyrosine-Protein Kinase Fyn; G6PD: Glucose-6-Phosphate Dehydrogenase; GCLC: Glutamate-Cysteine Ligase Catalytic Subunit; GCLM: Glutamate-Cysteine Ligase Modifier Subunit; GPX: Glutathione Peroxidase; GLU: Glutamate; GPX: Glutathione Peroxidases; GSSG: Glutathione Disulfide; GSH: Glutathione; GSK3: Glycogen Synthase Kinase-3; GSSG: Oxidized Glutathione; GST: Glutathione S-Transferase; GSTA2: Glutathione S-Transferase Alpha 2; GSTP: Glutathione S-Transferase P1; GSTP1: Glutathione S-Transferase Pi 1; H2AX: Histone Variant H 2 AX; HDAC4: Histone Deacetylase 4; HER2: Human Epidermal Growth Factor Receptor 2; Hif-1 α : Hypoxia Inducible Factor

1 Alpha Subunit; HO1: Heme Oxygenase 1; HMOX1: Heme Oxygenase (Decycling) 1; IDH1: Isocitrate Dehydrogenase (NADP(+)) 1; IKKB: Inhibitor Of Nuclear Factor Kappa-B Kinase Subunit Beta; IVR: Intervening Region; JNK1: Stress-Activated Protein Kinase Jnk-1; Keap1: Kelch-Like Erythroid Cell-Derived Protein 1; KRAS: Kirsten Rat Sarcoma; MAPK: Mitogen-Activated Protein Kinases; MDRP: Multiple Drug Resistance Protein; Mdrs: Multiple Drug Resistance; ME1: Malic Enzyme 1; Mirna: MicroRNA; Mitoq: Mitoquinone; Mnsod: Manganese Superoxide Dismutase; MRPS: Mitochondrial Ribosomal Protein; NADPH: Nicotinamide Adenine Dinucleotide Phosphate; Neh: Nrf-2-ECH Homology; Nfkβ: Nuclear Factor Kappa B; NQO1: NAD(P) H De Hydrogenase -Quinone 1; Nrf2: Nuclear Factor Erythroid 2-Related Factor 2; NTR: N-Terminal Region; P: Phosphorylation Site; P21: Protein21; P38: Protein 38; P62: Protein 38; PALB2: Partner and Localizer Of BRCA2; PERK: Proline-Rich Receptor-Like Protein Kinase; PGAMS: Phosphoglycerate Mutase; PGD: Phosphogluconate Dehydrogenase; PR: Progesterone Receptor; PI3K: Phosphatidylinositol 3-Kinase; PKC: Protein Kinase C; PPP: Pentose Phosphate Pathway; PTEN: Phosphatase and Tensin Homolog; RNF168: Ring Finger Protein 168; RNS: Reactive Nitrogen Species; ROS: Reactive Oxygen Species; Rxrα: Retinoid X Receptor α; SAHA: Suberoylanilide Hydroxamic Acid; SFN: Sulforaphane; SH: Sulfhydryl; Skp2: S-Phase Kinase-Associated Protein 2; Smaf: Small Musculo Aponeurotic Fibrosarcoma; SOD1: Superoxide Dismutase1; SQSTM: Sequestosome-1; SRXN: Sulfiredoxin; TALDO1: Transaldolase 1; TCA: Tricarboxylic Acid (TCA) Cycle; TKT: Transketolase; TNBC: Triple-Negative Breast Cancer; TPA- 12-O: Tetradecanoylphorbol-13-Acetate; TRX: Thioredoxin; TXN: Thioredoxin; TXNRD: Thioredoxin Reductase; UGT: UDP Glucuronosyl transferase; UPR: Unfolded Protein Response; WTX: Wilms Tumor Gene On X Chromosome; XBP1s: X-Box Binding Protein 1; Xct: Solute Carrier Family(XCT Channels); B-Trcp: B-Transducin Repeat-Containing Protein

INTRODUCTION

Nrf2 – a key transcription factor regulating oxidative stress in cells

NEF2 related factor (Nrf-2), a redox sensitive transcription factor, responds to oxidative and electrophilic stress signals by directing a wide variety of adaptive and cyto-protective responses through the transcriptional activation of genes containing an antioxidant response element (ARE) [1]. Nrf2 accelerates the transcription of genes involved in Phase-II detoxifying enzymes, xenobiotic transporters and other stress response proteins to combat the harmful effects of endo-and exogenous insults [2]. Hence, Nrf-2 is considered as a cytoprotective transcription factor with potential tumor suppressive effects [3]. Demonstrating this tumor suppressive effect, an animal model comparing the sensitivity of mice harboring Nrf2, with mice lacking Nrf2 to develop tumors reported that Nrf-2 deficient mice are more sensitive to carcinogenesis and formed extensive metastasis [2,4]. In normal conditions, Nrf-2's ability to promote transcription of ARE-containing genes is inhibited by Kelch-like erythroid cell-derived protein 1 (Keap-1). Keap-1 sequesters Nrf2 in the cytoplasm there by mark this protein for proteosomal degradation [1].

Structural features of nrf-2 and its negative regulator keap-1

Nrf2 belongs to a family of transcription factors, which also include Nrf-1, Nrf-2, Nrf-3, NF-E2, Bach1 and Bach 2 [5]. Structurally Nrf-2, in humans, is a 605 amino acids long protein that contains seven highly conserved Nrf-2-ECH Homology (Neh) domains [6] (Figure 1). Neh1 domain (amino acids 435-562) comprises a conserved CNC-bZIP region made up of DNA-binding peptide sequence and is essential for Nrf2 transcriptional activity. In addition, the Neh domains help in hetero dimerization with Maf proteins [7]. The N-terminal Neh2 domain (amino acids 17-87) contains two highly conserved Keap1 binding high-affinities ETGE motif and the lower-affinity DLG motif sites sequences [8]. The Neh 3-5 domains trans-activate various proteins by binding to the components of transcriptional apparatus [9]. For example, the C-terminal Neh3 domain (amino acids 562-605) transactivate Nrf-2. Likewise, Neh4 (amino acids 99-145) and Neh5 (amino acids 183-201) functions cooperatively to bind coactivators (Figure 1). The Neh6 domain amino acids 337-389) negatively control Nrf-2 and is responsible for Keap-1 independent regulation of Nrf-2 [10]. It contains two redox independent conserved peptide motifs, βDSGIS and DSAPGS, which are recognized by transducin repeat containing protein (β-TrCP) (Figure 1) [11]. However, these motifs are not recognized by Keap-1. This alternative pathway is enhanced by the phosphorylation of Nrf-2 by glycogen synthase kinase-3β, providing another mechanism for controlling Nrf-2 activity. Conversely, suppression of Nrf-2 is conferred by the interactions of Neh7 (amino acids 209-316) with the DNA-binding domain α. of retinoic X receptor Neh7 is responsible for direct protein-protein interaction between Nrf-2 and the DNA-binding domain of retinoid α (Rα) XR receptor [7]. Mechanistically, RXRα ligand b exarotene inhibit the expression of Nrf-2 target genes α and by promoting the Neh7 interaction between RXR domains. As a result Nrf-2 fails to bind to antioxidant response element (ARE) regions of the DNA and trigger the expression of antioxidant genes [12].

Keap-1 promotes the degradation of nrf-2

Under basal conditions, Keap-1 binds and promotes proteasomal degradation of Nrf-2 through an E3 ubiquitin ligase [11]. A rapid turnover of Nrf-2 prevents the unnecessary expression of Nrf-2 target genes. Keap-1 forms a homodimer *via* its BTB domain. The Neh2 domain of Nrf-2 contains two binding motifs: the high affinity ETGE and the low affinity DLG motifs, which binds to Keap-1 dimer [12]. Structurally, Keap-1 contains 24 free sulfhydryl (-SH) groups in its constituent cysteine residues. These highly reactive functional groups act as stress sensors and help in the modulation of cellular response [13]. For example, oxidative or electrophilic stress signals such as ROS and RNS, modify Keap-1 cysteine residues [14]. Under induced stress conditions, conformational changes on cysteine residues in Keap-1 disrupts binding at Nrf-2 DLG motif thereby promote the accumulation of newly synthesized Nrf-2 in the nucleus to activate ARE-containing genes [15].

Figure 1A

KEAP1

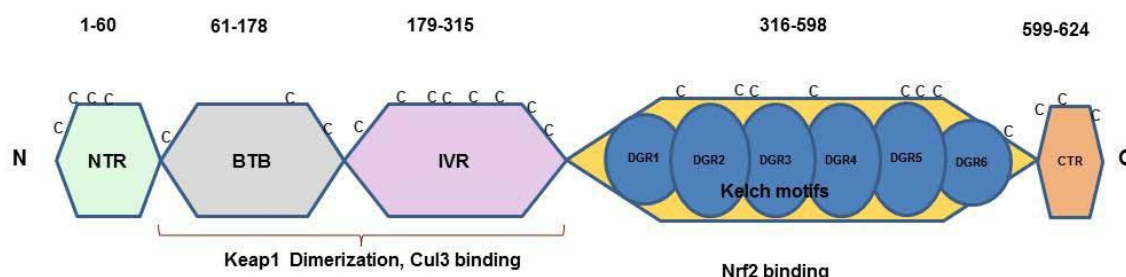


Figure 1B

Nrf2

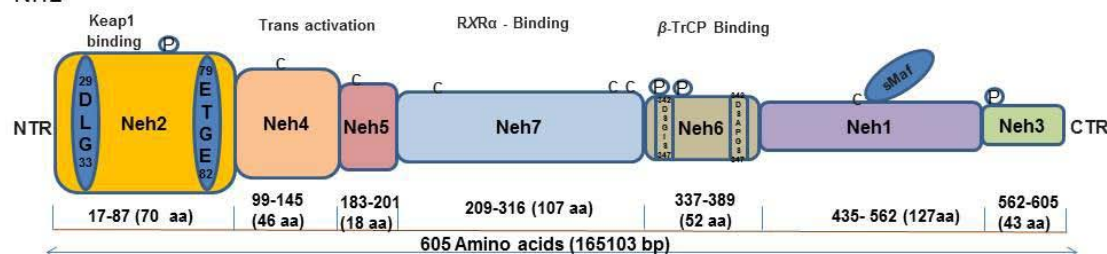


Figure 1 Schematic representation of the domain structure of Keap1 and Nrf2: **1A Structure of human Keap1 protein:** The 624 amino acids long Keap1 protein is divided into five major domains viz., N- terminal NTR (60 aa), Keap1 dimerizing BTB (118 aa), Cul3 binding IVR (137 aa), Nrf2 associating DGR sequences (283 aa) and C- terminal region CTR (26 aa). Cysteine residues present in the Keap1 helps in the substrate adaptor activity as well as in the regulation of the ubiquitination of Nrf2. **1B Structural features of human Nrf2:** 605 aminoacids long Nrf2 protein is divided into 7 domains. They are N-terminal Neh2 (70 aa),Neh4 (46 aa) ,Neh5 (18 aa), Neh7 (107 aa), Neh6 (52 aa),Neh1 (127 aa) and C terminal Neh3 (43 aa). Neh2 is responsible for binding of Nrf2 beta to Keap1, whereas Neh6 domain is responsible for -TrCP -mediated proteasomal degradation. In addition, Nrf2 has six highly conserved cysteine residues and four phosphorylation sites Ser40 (phosphorylated by PKC), Ser342/ Ser347 (phosphorylated by GSK3b) and Tyr568 (phosphorylated by Fyn).

Nrf-2 regulates genes containing ARE to mitigate the effects of oxidative stress

Cells respond to the stress signals induced by oxidative and electrophilic agent's exposure by promoting the translocation of Nrf2 in to nucleus. Nuclear Nrf-2 regulates the expression of over 200 genes that are involved in gene transcription, cytoprotection and lipid metabolism (Figure 2) [16]. While up-regulating the transcription of genes involved in combating oxidative stress, Nrf2 promotes the expression of proteins that produce NADPH and ATP [17]. For instance, Nrf-2 controls the expression of xCT subunit, which imports cysteine into cells along with glutamate-cysteine ligase catalytic (GCLC)-and modification (GCLM) subunits to catalyze the rate-limiting step in glutathione biosynthesis [17]. In addition to controlling the biosynthesis of glutathione, Nrf-2 also controls the expression of glutathione peroxidases (GPX), which converts reduced glutathione (GSH) to oxidized glutathione (GSSG) in the presence of hydrogen peroxide [18,19]. GPX enzyme helps to control the cellular oxidative stress by reducing toxic hydrogen peroxides (Figure 2) [20]. Besides controlling the glutathione based antioxidant systems, Nrf-2 also regulates the expression of oxidized protein thiols such as the expression of

cytosolic thioredoxin (TXN) 1, thioredoxin reductase (TXNRD)1 and sulfiredoxin (SRXN)1 thereby coordinate the regulation of glutathione homeostasis [7] (Table 1).

Nrf2 protect cells from carcinogens - the beneficial effect

Nrf-2 plays a critical role in protecting cells from carcinogens [21]. A prior study by Ramos-Gomez (2001) reported that Nrf-2-null mice are more likely to develop gastric neoplasia after exposure to benzo(a)pyrene compared with wild-type mice [22]. Similarly, a separate study showed higher tumor burden in the intestines of Nrf-2-deficient mice challenged with azoxymethane followed by dextran sodium sulfate compared with wild-type mice [23,24]. Moreover, the efficacy of chemo preventive agent 4-methyl-5-[2-pyrazinyl]-1,2-dithiole-3-thione (oltipraz) was less effective in *Nfe2l2* knockout mice, which is due to reduced induction of phase 2 detoxifying enzymes [25]. Additional studies have further confirmed these observations by demonstrating that: (a) Treatment of *Nfe2l2* knockout mice with 7,12-dimethylbenz(a)anthracene (DMBA), 12-O-tetradecanoylphorbol-13-acetate (TPA), or azoxymethane/dextran sulfate sodium (DSS) enhanced the incidence of carcinomas of skin, colon and rectum and or

Figure 2

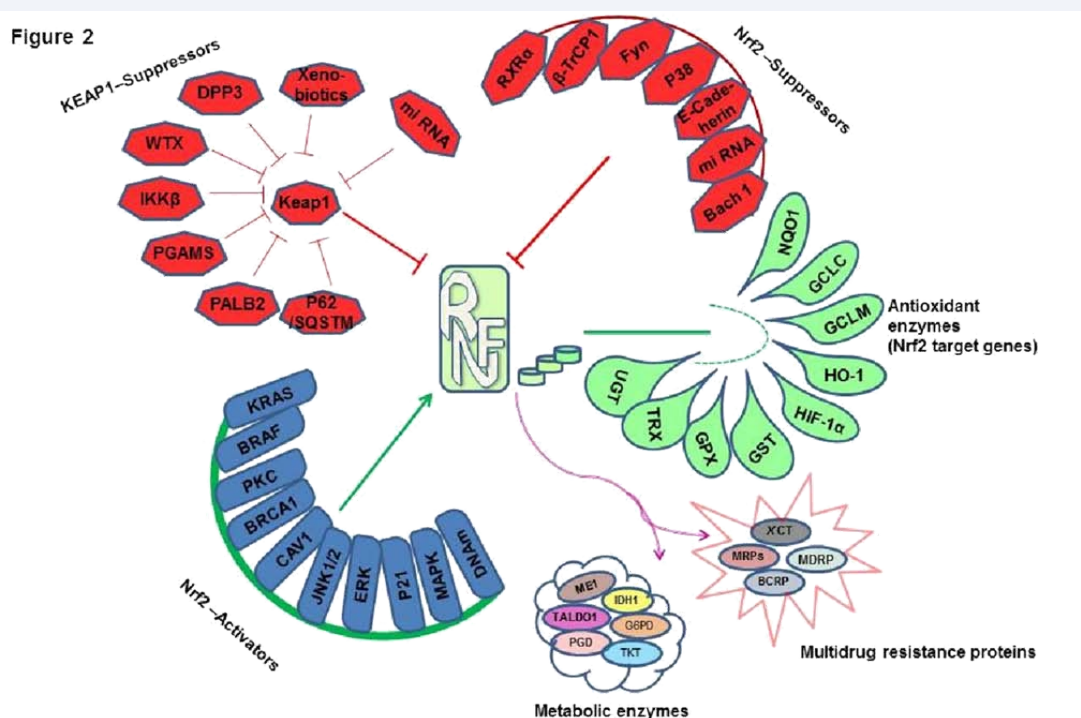


Figure 2 Key modulators and targets of Nrf2: Expression and activity of Nrf2 is regulated by upstream negative and positive modulators such as Keap1, and Fyn, Braf, PKC and p38MAPK etc... Active Nrf2 promotes the expression of genes involved in scavenging reactive oxygen species, exporting drugs (multidrug resistant proteins) and metabolic enzymes thereby enhance cell proliferation and make the cells resistant to treatment agents.

Table 1: List of key targets of Nrf2 and their functions in cells.

Name of the Target	NCBI- ID	V	References
	25827424	Glutamate-cysteine ligase catalytic (GCLc)- and modification (GCLm) subunits catalyze the rate-limiting step in glutathione biosynthesis, and helps to maintain cellular redox homeostasis	[17]
xCT	25827424	Cysteine-glutamate transporter assist in the transportation of cystein into cells along with glutamate-cysteine ligase catalytic	[17]
GPX	15342956	Glutathione peroxidase converts reduced glutathione (GSH) to oxidized glutathione (GSSG) in the presence of hydrogen peroxide and thereby maintain cellular redox homeostasis	[18]
TXN	26122708	Thioredoxin participate in dithiol-disulfide exchange reactions; reduces sulfenic acid in proteins and coordinate the regulation of glutathione homeostasis	[7]
TXNRD	26122708	Coordinate the regulation of glutathione homeostasis	[7]
HO1	20460467	Cleaves heme to produce biliverdin during heme catabolism.	[62]
NQO1	19608619	Reduces quinones to hydroquinones and prevents the one-electron reduction of quinones that would otherwise produce free radicals	[44]
SOD1	26475962	Superoxide dismutase scavenges superoxide radicals in the cytosol to protect cells from free radical damage.	[80]

mammary tissues [25,26]. Mechanistically, Nrf-2-mediated protection against chemical-induced carcinogenesis is due in part to its ability to reduce the amount of reactive oxygen species (ROS) and DNA damage in cells [26]. Constitutive Nrf-2 activation in cells is mediated by: (i) somatic mutations of Keap-1 or Nrf-2 [27] (ii) epigenetic silencing of the Keap-1 gene [28], (iii) aberrant accumulation of proteins that compete with Nrf-2 for Keap-1 binding [29] and (iv) oncogene mediated over expression of Nrf-2 [30]. For example, oncogenic activation of KRAS (KRASG12D), c-MYC (c-MYCERT12), and BRAF (BRAFFV619E) elevates

the transcript levels of NRF2 and its target gene expression. Hence, Nrf2 is a good cancer chemopreventive protein in cells and compounds that can elevate the expression of Nrf2 are potential chemopreventive agents [31]. Therefore, to identify effective chemopreventive agents several studies have isolated and characterized Nrf2 activators for their ability to prevent the induction of cancers. For example sulforaphane is a sulfur-containing compound isolated from broccoli [32]. Recent studies have demonstrated its potential for protecting the transformation of lung epithelial cells in to cancerous ones [33].

Dark side of Nrf-2

Although Nrf-2 activation can protect cells against a wide range of toxicants and stressors; aberrant activation of Nrf-2 has been associated with several types of cancers, especially carcinomas of breast and lung. Nrf2 promotes the survival of not only normal cells but also cancer cells. Therefore, accumulation of Nrf2 in cancer cells creates an environment conducive for cell growth and protect against oxidative stress, chemotherapeutic agents, and radiation therapy [34,35] Supporting this observation, nuclear accumulation of Nrf-2 has been demonstrated to correlate with poor prognosis and clinical outcome in carcinomas of breast and other organs [2]. Therefore, better understanding role of Nrf2 and its target genes regulation is required to develop effective strategies for treating breast cancer.

Role and regulation of nrf2 in breast cancer

Oxidative stress is one of the primary causes for promoting the transformation of a normal cell in to cancerous cells [7]. Like many other cancer types, even the carcinomas of breast develop due to unusually high levels of oxidative stress leading to the production of reactive oxygen and nitrogen species, which damage DNA, proteins and lipids thereby promoting cancerous transformation of normal cells [36-38]. Therefore, antioxidants that promote the expression of key proteins involved in the scavenging of free radicals are potential cancer preventive agents. Several studies have provided evidences to the belief that use of anticancer drugs increase the levels of reactive oxygen species (ROS) thereby cause damage to DNA [26]. Persistent accumulation of ROS is primarily due to a deficient antioxidant response mediated by Nrf-2 [39]. As a result elevated ROS reduces H2AX protein levels by promoting its association with E3 ubiquitin ligase RNF168 [40]. For example, a report identified that chemotherapy increases ROS and reduces H2AX protein levels in Triple-Negative Breast Cancer (TNBC) patients [26]. Reduced H2AX proteins fail to launch effective DNA damage response (DDR), and make the tumor cells more sensitive to various therapeutic agents [41]. For example, partial inactivation of H2AX by siRNA is sufficient to sensitize TNBC cells to cisplatin [26]. Since, reduced H2AX increases DNA damage and sensitizes tumor tissue to anticancer therapies; it is a good predictor of the

therapeutic efficiency of pharmacological agents as well as the survival of TNBC patients.

In some breast cancers, hormone receptors such as HER2 can also increase the expression of Nrf-2 and its target genes [29]. HER2 over expression results in p62 accumulation, which helps in the activation of Akt in mammary cells [29]. In addition, p62 binds to Keap1 thereby disrupts its interaction with Nrf2 [42]. Furthermore, expression and activation of HER2 confer resistance of cancer cells to chemotherapeutic drugs, as it increases the binding of Nrf-2 to DNA and activate ARE-mediated transcription [43]. HER2 induces resistance in breast cancers through (a) the induction of drug efflux pumps including ABCB1 and ABCC3 [44]; (b) the enhancement of the expression of drug metabolizing proteins such as glutathione S-transferase P1 (GSTP1) and cytochrome P450 3A4 (CYP3A4) [45]; and (c) the stimulation of the expressions of cell survival proteins such as Survivin [46]. These tumor-promoting activities of HER2 are primarily mediated by the induction of Nrf2 in many cancers including the carcinomas of breast [43,47] (Table 2).

Modulation of Nrf-2 expression and the fate of breast cancer cells

Oxidative stress is one of the primary causes for promoting the transformation of a normal cell in to cancerous cells [7]. Like many other cancer types, even the carcinomas of breast also develop due to unusually high levels of oxidative stress leading to the production of reactive oxygen and nitrogen species, which damage DNA, proteins and lipids thereby promoting cancerous transformation of normal cells [36-38]. Therefore, antioxidants that promote the expression of key proteins involved in the scavenging of free radicals are potential cancer preventive agents. For example, a recent study has shown that phytochemicals such as phenylethylisothiocyanate and sulforaphane upregulates the expression of Nrf2 thereby prevent the formation of tumorigenic cells [32]. However, once advanced, the cancer cells use the antioxidant defense mechanisms for their proliferation and drug resistance [43]. For instance, elevated Nrf2 has been identified in many advanced tumors that include carcinomas of breast, lung and colon and rectum. A study has revealed that Nrf-2 activation conferred resistance to chemotherapeutic drugs such

Table 2: List of targets that are regulated by NRF2 through intermediary proteins.

Name of the Target	NCBI -ID	Role in Cancer	References
Production of NADPH	22439925	Reducing power in the form of NADPH (required for protection from ROS and fatty acid biosynthesis)	[75]
H2AX	27006338	H2AX proteins fail to launch effective DNA damage response (DDR), and make the tumor cells more sensitive to various therapeutic agents	[26]
ABCB1	22280939	Drug resistance through drug efflux pumps	[46]
ABCC3	19608619	Biliary transport/hepatic excretion; involved in the extrusion of certain anti-cancer drugs and implicated in multidrug resistance	[44]
GSTP1	22331464	Drug metabolizing and detoxifying enzymes' regulatory functions such as JNK signaling pathway.	[58]
CYP3A4	16550168	Participate in the inactivation and activation of anticancer drugs. Play a key role in the Phase-I dependent metabolism of drugs and other xenobiotics.	[45]
PPP	23921124	Metabolic shift: Carbon flux toward the pentose phosphate pathway (PPP) and the tricarboxylic acid (TCA) cycle	[74]
Mrp1	11279022	Confers multidrug resistance on tumor cells by reducing drug accumulation by active efflux	[78]

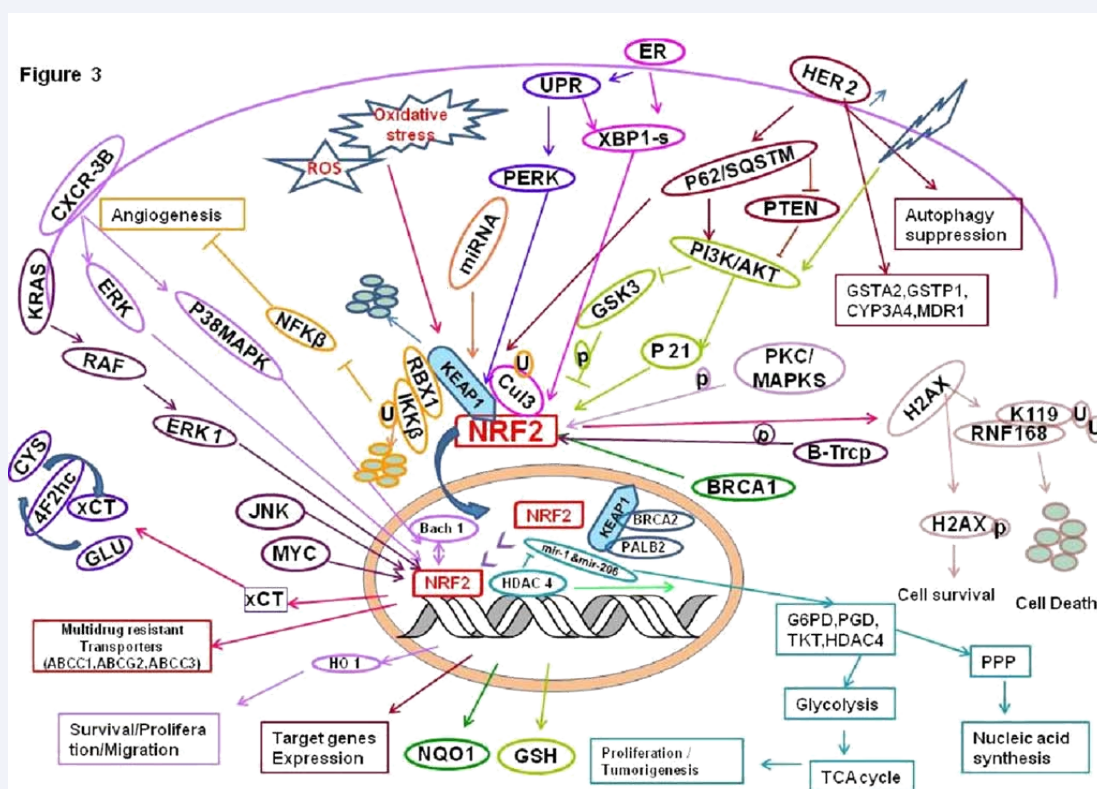


Figure 3 Schematic representation demonstrating various signaling cascades modulated by- or modulating- Nrf2 pathway in cancers. Nrf2 is involved in controlling cell proliferation, apoptosis, autophagy, angiogenesis, drug resistance and metabolism in cancers. While cell surface molecules like KRas, CXCR3, ER and HER2, activates Nrf2, proteins like Keap1, GSK3B and IKKB promote the degradation of Nrf2 in cancer cells.

as 5-fluorouracil, cisplatin and paclitaxel [2]. Likewise activation of Nrf-2-ARE pathway in MCF7 breast cancer cell line provided resistance to tamoxifen when incubated for longer time period [48]. Further, a separate study by Kim et al. (2008), reported that expression of Nrf-2 dependent antioxidant proteins was increased in tamoxifen resistant breast carcinoma cells, and knocking down Nrf2 partially lifted the resistance induced to tamoxifen treatment [48]. Demonstrating the oncogenic function of Nrf2, many recent studies have shown that targeted inhibition of Nrf2 using siRNA mediated knockdown retarded the tumor cells proliferation and made the cells sensitive to chemotherapeutic agents as well as radiation treatment [34]. Therefore, many studies now point that Nrf2 has dual functions in cancers, while very low levels fail to prevent the transformation of normal cells to cancer cells, very high Nrf2 levels present in advanced tumors provide protection to cells by scavenging ROS thereby act as oncogenic protein. Hence, one should execute caution while selecting the Nrf2 modulators for treating cancers. While Nrf2 activators are more beneficial for preventing cancers, the inhibitors of Nrf2 are good chemotherapeutic agents. While mitigating the ROS levels through the induction of scavenging enzymes, maintenance of appropriate glutathione levels in the cells is also important to protect cells from ROS-induced damage [49]. Cellular glutathione levels are regulated by glutathione synthesizing enzymes as well as the enzymes oxidizing this molecule. For example, oncogenic PI3K/Akt stimulates glutathione (GSH) biosynthesis by activating key enzymes involved in glutathione synthesis [49]. Mechanistically, Akt stabilizes Nrf-2 by promoting the

accumulation of p21Cip1/WAF1 as well as by inhibiting GSK-3 [50]. Accumulated p21 directly binds to Nrf-2 and disrupt the Keap1-Nrf-2 interaction, thereby activate Nrf2. In the other hand, elevated GSK-3 phosphorylates and directs Nrf-2 towards ubiquitination and proteasomal degradation [51].

In some cancers, hormone receptors such as HER2 can also increase the expression of Nrf-2 and its target genes [29]. HER2 over expression results in p62 accumulation, which in turn helps in the activation of Akt in mammary cells [29]. In addition, p62 binds to Keap1 thereby disrupts its interaction with Nrf2 [42]. Furthermore, expression and activation of HER2 confer resistance of cancer cells to chemotherapeutic drugs, as it increases the binding of Nrf-2 to DNA and activate ARE-mediated transcription [43]. In summary, HER2 induces resistance in breast cancers through (a) the induction of drug efflux pumps including ABCB1 and ABCC3; (b) the enhancement of the expression of drug metabolizing proteins such as glutathione S-transferase P1 (GSTP1) and cytochrome P450 3A4 (CYP3A4); and (c) the stimulation of the expressions of cell survival proteins such as Survivin [46]. These tumor-promoting activities of HER2 are primarily mediated by the induction of Nrf2 in many cancers including the carcinomas of breast [43,47].

Other proteins that regulate the stability of Nrf2 include (1) glycogen synthase kinase 3 β (GSK3 β); (2) β -transducin repeat-containing protein (β -TrCP); and (3) CR6-interacting factor-1 (CRIF1) [52]. These proteins down-regulate Nrf2 by proteasomal degradation in cells. Protein-protein interactions

do play important role in the regulation of Nrf2 levels in the cells [43]. For example, KEAP1, p21, Wilms tumor gene on X chromosome (WTX), p62, (PALB2), Dipeptidyl peptidase III (DPP3), and Breast cancer susceptibility gene 1 (BRCA1) and HER2 interacts with NRF2 in HER2-positive breast cancer cells thereby promote its degradation [42,50,53-55]. A study has shown that BRCA1 regulates the transcription of Nrf2. Supporting this, 5-10% of breast cancer cases in the Western world harbor a loss-of-function mutation in the tumor suppressor gene BRCA1. In addition, BRCA1-deficient mice showed low levels of Nrf2-regulated antioxidant enzymes and accumulate ROS that impair survival *in vivo* [56]. PALB2, a major BRCA2 binding partner, also interacts with Keap-1 through its highly conserved ETGE-type Keap-1 binding motif to promote the nuclear localization of Nrf-2 [57]. In addition, in the nucleus, PALB2 prevents Keap-1 from binding to Nrf-2 thereby promote the nuclear concentration of Nrf2 [58]. PALB2 is mutated in breast cancers [59]. It controls the intra nuclear localization, stability and DNA repair function of BRCA2 [43]. A separate study has shown that loss of PALB2 lead to increased ROS level and reduced Nrf-2 transcriptional activity without affecting its total abundance in the cell [58].

CXCR3-B is another regulator of Nrf2 in cancers [60]. CXCR3-B promotes the activation of p38 MAPK thereby inhibit ERK1/2 [61]. As a result Bach-2 localizes to nucleus and promote the export of Nrf2 from nucleus. CXCR3-B mediates growth-inhibitory signals in human breast cancer cells by down regulating the expression of Heme Oxygenase-1 (HO1) [60,62]. Breast cancer patients with minimal Nrf-2 expression have a lower incidence of relapse when compared to those with high Nrf-2 expression in the PR+/ER+ or TNBC groups [27].

The scaffold protein caveolin-1 is another regulator of Nrf2 in cancers [63]. Caveolin-1 (Cav-1) is located in the lipid raft domains of the cellular plasma membrane and forms a strong ternary complex directly with both Keap1 and Nrf2 [64]. Fluctuations in the levels of H2O2 lead to the release of Nrf2/Keap1 from Cav-1 and the dissociation of Nrf-2 from Keap-1 [65]. Cav-1 potentially reduces MnSOD expression through negative regulation of Nrf-2 activity in breast cancer. Cav-1 and MnSOD expression are major determinants of metabolic changes that support tumor progression. Aggressive breast cancers express low Cav-1 and high MnSOD [64]. Studies have proved that lack of Cav-1 suppresses glycolysis and increases mitochondria-dependent ATP production. Furthermore, degradation of Cav-1 suppresses Nrf2 activity, which leads to upregulation of MnSOD and the activation of AMPK and glycolytic switch [64]. Keap-1 regulates several cellular pathways that are involved in carcinogenesis [65]. Ikk β is one of the Keap-1 substrates for ubiquitination [66]. Under β oxidative is stress, Ikk detached from Keap-1 and initiat κ B regulates the activation genes, resulting of Nf in increased growth, proliferation and anti-apoptosis [7]. This contributes towards cell survival and tumor progression. In addition, oxidative stress promotes Keap-1, which degrades anti-apoptotic proteins Bcl2 and Bcl-xL thereby modulates the intrinsic apoptotic pathway. Keap-1 act as a tumor suppressor gene and loss of Keap-1 function confers tumorigenic potential to the cells [4]. Supporting this, a study identified KEAP1 mutation C23Y in cancers [56]. Mutant C23Y Keap1 lacks the ability to

inhibit Nrf2 as it fails to make interactions with NRF2, which leads to increased protein stability and antioxidant activity in human breast cancers [67].

Regulation of Nrf2 expression and its transcriptional activity is a well-controlled process. Like many other proteins, the expression of Nrf2 is regulated at genetic and epigenetic levels [67,68]. Whereas the chromosomal aberrations and mutations represent the regulation at genetic level; the methylation, histone acetylation and microRNA-mediated genetic changes contribute for the epigenetic regulatory mechanisms [28,69]. In addition, the expression of Nrf2 is also regulated by changes in the translation and posttranslational modifications, cellular localization and protein-protein interactions [7]. Even though regulation of Nrf2 expression at genetic level is well explored in lung cancers, not much is known about this aspect in breast cancers [2]. Hence, further studies are warranted to determine whether Nrf2 expression is regulated at genetic level, if so what are the mechanisms being operated in the breast cancers. In the following sections, we have given overview about the epigenetic mechanisms regulating Nrf2 expression and their changes in carcinomas of the breast.

Epigenetic regulation

MicroRNA mediated gene regulation is one of the epigenetic mechanisms widely studied in breast cancers [16,70]. miRs are endogenous, single stranded non coding RNAs of 18-22 nucleotides length [28]. They suppress gene expression by binding to miR response elements within 3'-UTRs of target mRNAs [66]. Mechanistically, miRNAs inhibit target gene expression through degradation of mRNA or inhibition of translational process. miRNA-dependent translation suppression of target gene expression involve: (1) Repression of 7-methyl-guanosine (m7G) cap-dependent mRNA translation, (2) prevention of the joining of 60S subunit, (3) repression by translation termination, and (4) possible involvement in the proteolysis of nascent polypeptide chains [66]. For example, epigenetic silencing of miR-200a contributes to dysregulation of Keap1 [28]. A study has shown that treatment of breast cancer cells with HDAC inhibitor SAHA restored miR-200a thereby down regulated Keap1 [28]. Down-regulated Keap1 promotes the reactivation of Nrf2-dependent antioxidant pathways in breast cancers (Figure 4) [28]. MiR-146 is another micro RNA altered frequently in breast cancer cell lines [68]. It consists of miR-146a and -146b. miR-146 is a critical factor implicated in cell proliferation and metastasis [71]. Mechanistically, miR-146a suppresses cell growth by inducing cellular apoptosis and by down-modulating epidermal growth factor receptor. In ER-positive breast cancer cells miR-146a/b is induced by FOXP3 [68]. However, in TNBCs it is not known how and which mechanism is regulating the expression of miR-146a or 146b. Further studies are warranted to determine the key factors regulating the expression of miR-146a and mir-146b as these microRNAs are known to suppress metastasis of breast cancer cells [68]. Likewise, miRNAs can also regulate the function of transcription factors such as NF- κ B and Nrf2 in breast cancers [72]. For example, miR-28 is an important microRNA regulating the expression of Nrf2. It binds to the 3'UTR of Nrf2 mRNA and triggers its degradation [69]. Similarly, miR-93 also binds to 3'-UTRs in thereby promote the down-modulation of Nrf2-induced target genes in breast cancers [73].

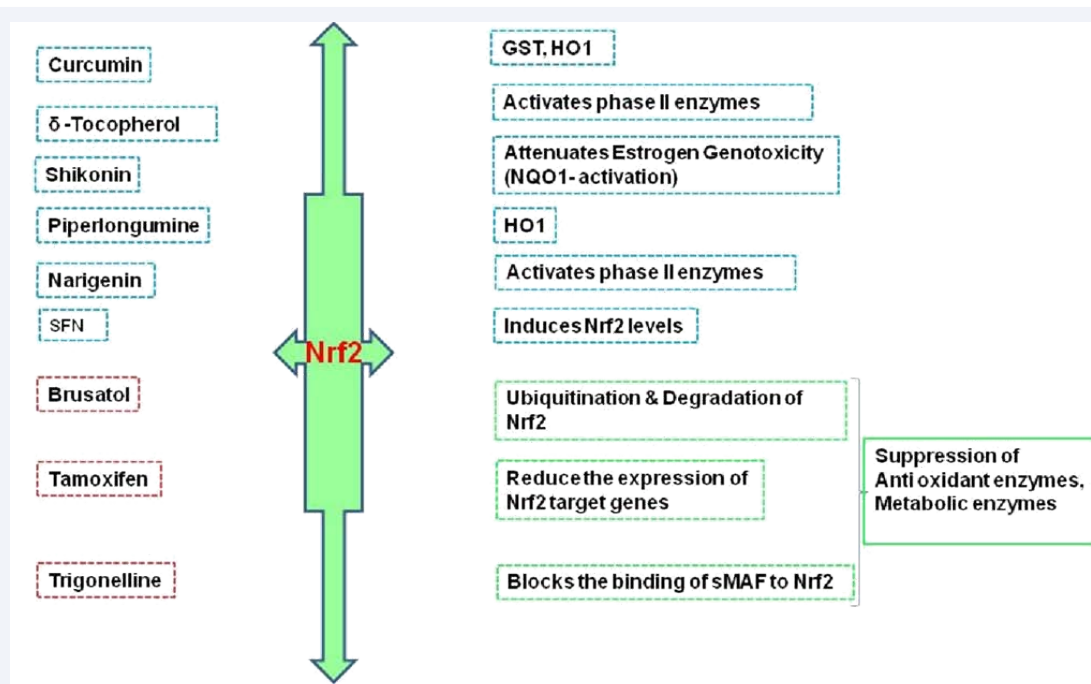


Figure 4 Pharmacological agents targeting Nrf2 signaling: Naturally occurring Curcumin, δ -Tocopherol, Shikonin, Piperlongumine, Narigenin and SFN activate Nrf2 to provide protection to cells from oxidative stress induced death. However, the inhibitors of Nrf2 such as Brusatol, Tamoxifen, Trigonelline suppresses Nrf2 by promoting its degradation, which ultimately results in the down-regulation of target genes.

While many microRNAs regulate the expression of NRF2, Nrf2 will also regulate the expression of several genes by modulating the expression of proteins. For instance, Nrf2 control the key enzymes of TCA cycle. Mechanistically, miR-1 and miR-206 control the carbon flux toward the pentose phosphate pathway (PPP) and the tricarboxylic acid (TCA) cycle (Figure 4 (included as supplementary data)) [74]. In general, cancer cells utilize glucose more extensively compared to normal cells to meet various metabolic needs that include supply of (a) energy in the form of ATP and NADH; (b) carbons for the synthesis of ribose (required for nucleic acids synthesis); (c) reducing power in the form of NADPH (required for protection from ROS and fatty acid biosynthesis) [75]. Therefore, it is important for cancer cells to effectively manage the carbon flux. Stable activation of NRF2 signaling in cancer cells attenuate miR-1 and miR-206 expression, leading to enhanced expression of PPP genes. Proof-of-principle studies evaluating the over expression of miR-1 and miR-206 showed a significant decrease in the expression of metabolic genes and impaired NADPH production, ribose synthesis, and tumor growth in mice [74]. Loss of NRF2 reduced the expression of redox-sensitive histone deacetylase, HDAC4, resulting in increased expression of miR-1 and miR-206 [74].

***In vitro* & *in vivo* studies demonstrating the role of nrf2 in cancers**

Elevated expression of Nrf2 has been reported in various cancer cell lines [76]. For example, a study demonstrated that in breast cancer cells MCF7 and SK-BR-3 nuclear Nrf-2 levels were decreased significantly when the cells were transfected with Nrf-2 siRNA [2]. In addition, targeted inhibition of Nrf2 reduced the mRNA levels of cytoprotective gene NQO1 in MCF7 and SK-BR-3

(MCF7, 24% and 22%). Even other cytoprotective genes such as HO-1 were also lowered by 25% [2]. Among different Nrf-2 target genes only Mrp1 has two ARE motifs in the promoter region. A high correlation between Mrp1 and Nrf-2 expression was reported in different tumor types. siRNA mediated knockdown of Nrf2 showed decreased expression of Nrf2 and MRP1 by 50% indicating that Mrp1 is regulated by Nrf-2 pathway [77,78]. In a separate study Habib E et al., has shown that over expression of Nrf-2 in MCF-7 cells up-regulate the activity of the xCT. In contrast, over expression of Keap-1 repressed promoter activity and decreased xCT level [17].

In mice bearing 4T1 breast carcinoma tumors, administration of tamoxifen, a widely used anti-breast cancer agent for treating ER alpha positive individuals, showed decreased tumor growth but increased expression of Nrf-2, and its target antioxidant genes NQO1, HMOX1, SOD1 [44,79,80]. More over the multidrug resistant transporters ABCC1, ABCG2, ABCC3 showed an enhanced activity [81]. Tamoxifen at clinical concentrations, accumulated in tumors during therapy, increases oxidative stress in ER α -positive and ER-negative breast cancer cells, resulting in cell death [82]. Tamoxifen-induced oxidative stress, increased the accumulation of the transcription factor- Nrf-2, which activates the antioxidant response element (ARE) and this contributes to chemo-resistance [2,79,83]. However, clinical resistance to tamoxifen, as demonstrated by recurrence or progression on therapy, is frequent and proceeds to death from metastases. Breast cancer cells respond to tamoxifen-induced oxidation by increasing Nrf-2 expression and subsequent activation of the ARE [83]. This increases the transcription of antioxidant genes and multidrug resistance transporters. Therefore, elevated Nrf-2, ABCC1, ABCC3 plus NAD (P)H dehydrogenase quinone-1 in breast

tumors indicates poor survival of individuals after tamoxifen therapy [84]. Therefore, overcoming tamoxifen-induced activation of the ARE could increase the efficacy of tamoxifen in treating breast cancer. Inhibition of Nrf-2 with trigonelline is one approach for overcoming the tamoxifen-induced resistance [79]. For example, trigonelline significantly decreases CDDP resistance in both Nrf-2 expressing MCF7 and MDA-MB-231 cells under hypoxia [79]. Nrf2 activity was decreased in the treatment group of CDDP and Trigonelline combination compared to the control or CDDP alone group. Nrf-2, an important regulator of drug sensitivity of a cell, is a potential target for treating drug resistant breast tumors, especially under hypoxia microenvironment [85]. Nrf-2 inhibition increases the chemotherapeutic sensitivity and decreased the tumor size significantly.

Modulators of Nrf2 in the prevention and treatment of cancers

Since many *in vitro* and preclinical *in vivo* studies have demonstrated the role of Nrf2 in tumor development, now, strategies have been designed to treat tumors by targeting Nrf2. Several pharmacological agents with increased safety and efficacy have been developed and efficacy tested. Therefore, in this section of the review, we have made an attempt to address the recent developments testing the pharmacological modulators of Nrf2 (Figure 4).

Modulators of Nrf-2 and Keap-1

Inhibitors of Nrf2 expression and activity:

Brusatol: Brusatol is a derivative containing quassinoid skeleton. It is isolated from *Brucea javanica*, a plant distributed widely in Asia and is used traditionally in Chinese medicine for treating cancer, malaria and inflammatory disorders. Adedamola Olayanju et al. (2015), demonstrated that brusatol enhances the phosphorylation of p38 MAPK, AKT, ERK1/2, and JNK1/2 while inhibiting Nrf2 [86]. Nrf2 inhibitory properties of Brusatol are mediated by the ubiquitination followed by the degradation of ubiquitinated Nrf. Brusatol enhanced intracellular ROS and sensitized mammospheres made up of MCF7 and MDA-MB-231 to taxol. Furthermore, treatment of cells with Brusatol reduced the anchorage-independent growth [1,87] (Table 3 (included as supplementary data)).

Luteolin and Chrysin: These Nrf2 inhibiting flavones that differ in their number of hydroxyl groups in the B ring have been isolated in high concentrations from celery, green pepper, and parsley. They have been tested for inhibiting cancer, and inflammatory disease. Mechanistically, the model compound luteolin inhibits Nrf2 by promoting the degradation of Nrf2 mRNA [88]. Luteolin sensitizes cancer cells to anticancer drugs oxaliplatin, bleomycin, and doxorubicin thereby induces apoptotic death. Chrysin (5,7-dihydroxyflavone) is another Nrf2 inhibitor isolated from, Honey, and Propolis. Like luteolin, it also inhibits the growth of cancer cells and helps in treating inflammatory disorders [89]. Chrysin significantly reduces Nrf2 expression by down regulating the PI3K-Akt and ERK pathways. Further, chrysin restores chemo sensitivity by down regulating the Nrf2-target genes such as HO-1, AKR1B10, and MRP5 in cancers [31,90].

Trigonelline (TRG): is a pyridine alkaloid commonly found in *Trigonella foenumgraecum* L. (fenugreek) seeds and coffee beans. Whereas one study reported that trigonelline upregulates the expression of PKCa and Raf/ERK pathway members thereby decreases cellular Nrf2 [91] another study showed that trigonellin could block Nrf2-sMaf-DNA complex. Inhibition Nrf2 activation by trigonellin down regulated the expression of cellular antioxidant machinery, SOD, catalase, and glutathione peroxidase expression [31]. Furthermore, Trigonelline sensitizes cell lines to anticancer drugs and tumor necrosis factor-related apoptosis inducing ligand (TRAIL) treatment [92].

Ochratoxin-A: A compound from *Aspergillus* and *Penicillium* inhibits Nrf2 through the induction of miRNA. OTA induced miR-132 in LLC-PK1 cells thereby decreased the expression of Nrf2. Targeted inhibition of miR-132 using gene knockdown procedures using antigomin prevented the decrease of Nrf2 mRNA upon OTA exposure [93] (Table 3 (included as supplementary data)).

Activators of Nrf2 expression and activity:

Shikonin: Shikonin is a bioactive agent extracted from Shikon plant, which is a major component of Zicao. Zicao is a key ingredient in Chinese herbal medicine [94]. Shikonin induces Nrf-2 dependent NQO1 transcription and attenuates estrogen genotoxicity in estrogen-dependent human breast cancer cells. It inhibits the interaction of transcriptional α and SIRT1 repressors at the ER NQO1 promoter site [82]. Shikonin not only inhibits transformation of normal mammary epithelial cells in to malignant ones but also helps in the prevention of estrogen-dependent tumor formation.

Sulforaphane (SFN): Sulforaphane is an isothiocyanate derived from the glucosinolate glucoraphanin. It is abundant in cruciferous vegetables. SFN can modulate gene expression through the interaction with Keap1 Cysteine 151 residue, which disrupts its function and allows the nuclear accumulation of Nrf2. Enhanced transcription of Nrf2 and its target genes provokes a strong cytoprotective response, which enhances resistance to anticancer agents [95,96].

Mitoquinone (MitoQ): Mitoquinone is a synthetic, redox-active ubiquinone. It induces autophagy as well as apoptosis in human breast cancer cells. Due to its ability to accumulate in mitochondria, MitoQ induces ROS, which leads to Keap1 oxidation followed by degradation. Degradation of Keap1 promotes the activation of Nrf2, which subsequently induces the expression of antioxidant genes and P62. Studies have also shown that even the p62 can bind Keap1 and activate Nrf2 further resulting in a positive feedback loop. MitoQ is 30-fold more cytotoxic to breast cancer cells than to healthy mammary cells [97]. Inhibition of cancer cell growth is associated with G1/S cell cycle arrest and phosphorylation of the checkpoint kinases Chk1 and Chk2.

Curcumin (CUR): Curcumin is a yellow bioactive component present in Indian spice turmeric. Curcumin inactivate the Nrf2-Keap1 complex, thereby enhance the binding of Nrf2 to ARE sequence containing genes. Mechanistically, curcumin induces the expression of Nrf-2 in breast cancers by decreasing Fen1 promoter activity. It inhibits the proliferation of breast cancer cells lacking ER/HER2 expression [90]. In addition, a recent study has shown that curcumin inhibits MCF-7 cell proliferation

through miR-19 mediated induction of p27, especially in MDA-MB-231 breast cancer cells [98].

Tocopherol: It is a member of the vitamin-E family and is predominantly in corn oil and soybean oil. Tocopherol activates Nrf2 by increasing its nuclear translocation/DNA binding activity. In addition, it increases cysteine availability leading to glutathione (GSH) synthesis and the levels of downstream Phase-II detoxifying and antioxidant enzymes TXN, GCLm, GSTm1, GPx and HO-1 NQO1[99,100].

Endogenous Nrf2 activators: Thiol-reactive electrophilic metabolites Acrolein (15-deoxy-1214-prostaglandin J2), Dopa-O-quinone, Fumarate, 4-Hydroxynonenal, and Maleylacetoacetate are the key endogenous molecules that can regulate the expression of Nrf2 by modifying cysteine residues in Keap1 (Table 4 (included as supplementary data)).

Synthetic Nrf2 activators: Synthetic compounds such as Dexamethasone, Protandim, Methazolamide, Nifedipine, Ambrisentan, Amlodipine, and Sitaxentan, which are the drugs commonly used to treat high illnesses, have been shown to activate Nrf2. However, the effect of these synthetic molecules on Nrf2 activation is an “off-target” effect regulated through phosphoinositide 3-kinase (PI3K) activation (Table 4 (included as supplementary data)).

CONCLUSION

In conclusion, the antioxidant regulator Nrf2 has a key role in breast cancer development and drug resistance. While Nrf2 activators help in the prevention of transformation of normal cells in to cancer cells, the inhibitors of Nrf2 retard the tumor growth and sensitize treatment-resistant cancer cells to various chemotherapeutic agents and radiation. Therefore, it is important to determine the stage (early or advanced) of breast cancer before selecting Nrf2 inhibiting pharmacological agents for treatment. In addition, it is also important to determine the receptor (ER, PR, and HER2) expression status as the levels of Nrf2 and its target genes exhibit differential expression pattern with differences in receptor type. Further studies are warranted to develop more effective Nrf2 modulators for treating breast cancers as the currently available inhibitors have not either evaluated in clinical trials or failed to show effective tumor inhibition in clinical trials.

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