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Review

Targets and mechanisms of sulforaphane derivatives obtained from cruciferous plants with special focus on breast cancer – contradictory effects and future perspectives

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ABSTRACT

Breast cancer is the most common type of cancer among women. Therefore, discovery of new and effective drugs with fewer side effects is necessary to treat it. Sulforaphane (SFN) is an organosulfur compound obtained from cruciferous plants, such as broccoli and mustard, and it has the potential to treat breast cancer. Hence, it is vital to find out how SFN targets certain genes and cellular pathways in treating breast cancer. In this review, molecular targets and cellular pathways of SFN are described. Studies have shown SFN inhibits cell proliferation, causes apoptosis, stops cell cycle and has anti-oxidant activities. Increasing reactive oxygen species (ROS) produces oxidative stress, activates inflammatory transcription factors, and these result in inflammation leading to cancer. Increasing anti-oxidant potential of cells and discovering new targets to reduce ROS creation reduces oxidative stress and it eventually reduces cancer risks. In short, SFN effectively affects histone deacetylases involved in chromatin remodeling, gene expression, and Nrf2 anti-oxidant signaling. This review points to the potential of SFN to treat breast cancer as well as the importance of other new cruciferous compounds, derived from and isolated from mustard, to target Keap1 and Akt, two key regulators of cellular homeostasis.

1. Introduction

Breast cancer is the most common type of cancer affecting women worldwide. According to epidemiological studies, cruciferous vegetables have protective effects on breast cancer [1]. One of the active compounds in them is glucosinolate (GLS) [2,3]. These are chemical compounds, all of which are made up of β-D-thioglucose, sulfonated oxime, and a side chain derived from branched amino acids or methionine, phenylalanine, and tryptophan amino acids. The GLS is hydrolyzed with an enzyme to form isothiocyanate (ITC) and it is biologically activated [4]. One of the ITC compounds is sulforaphane (SFN) (PubChem CID: 5350; molecular formula: $C_6H_{11}NOS_2$; molecular weight: 177.28 g/mol; Table 1; Fig. 1) that is obtained from hydrolysis of glucoraphanin (GFN) [5]. The SFN contents of European *Brassica* varieties have been previously studied and the results showed the content of SFN (mg/g dried weight) differ among varieties and their age, such as between 3-day-old and 9-day-old seedlings. The amount of SFN was the highest in three-day seedlings of *Brassica oleracea* L. cultivars San Martino, Primor (var. *italica*), and Ramoso Calabrese (2.21, 1.82, and 1.74 mg/g dried weight respectively). It was found SFN content was lower in 9-day-old seedlings of all cultivars indicating the developmental stage of each plant affects its SFN concentration [6].

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Table 1
Chemical information of sulforaphane and related key compounds extracted from cruciferous plants. These compounds along with SFN can be further examined in breast cancer research.

Chemical Names (abbreviation in the text)	PubChem CID	Molecular Formula	Molecular Weight (g/mol)	LogP[a]	TPSA[b] (Å^2)
Sulforaphane (SFN)	5350	$C_6H_{11}NOS_2$	177.3	1.4	80.7
Phenylisothiocyanate (PITC)	7673	C_7H_5NS	135.19	3.28	44.4
Mustard (*Brassica hirta and Brassica nigra*)	9605256	$C_{25}H_{33}N_3O_{11}S_3$	647.7	−1.1	311
2-Propenyl glucosinolate (S2)	5486549	$C_{10}H_{17}NO_9S_2$	359.4	?	200
Sinigrin	23682211	$C_{10}H_{16}KNO_9S_2$	397.5	2.8	203
4-Hydroxybenzyl ITC (HBITC)	160611	C_8H_7NOS	165.21	0	64.7
4-Hydroxybenzylamine (HBA)	97472	C_7H_9NO	123.15		46.2
Brassica oleracea Alkaloid (BOA)	100978913	$C_{13}H_9N_3O_2S$	271.3	2.1	93.3
D,L-Sulforaphane Boc-L-cysteine (SFNCys)	45040446	$C_{14}H_{26}N_2O_5S_3$	398.6	1.2	181
L- Sulforaphene (SFE)	11620	$C_6H_9NOS_2$	175.3	1.5	80.7
Phenethyl isothiocyanate (PEITC)	16741	C_9H_9NS	163.24	3.5	44.4

[a]Octanol/Water Partition Coefficient.
[b]Topological Polar Surface Area.

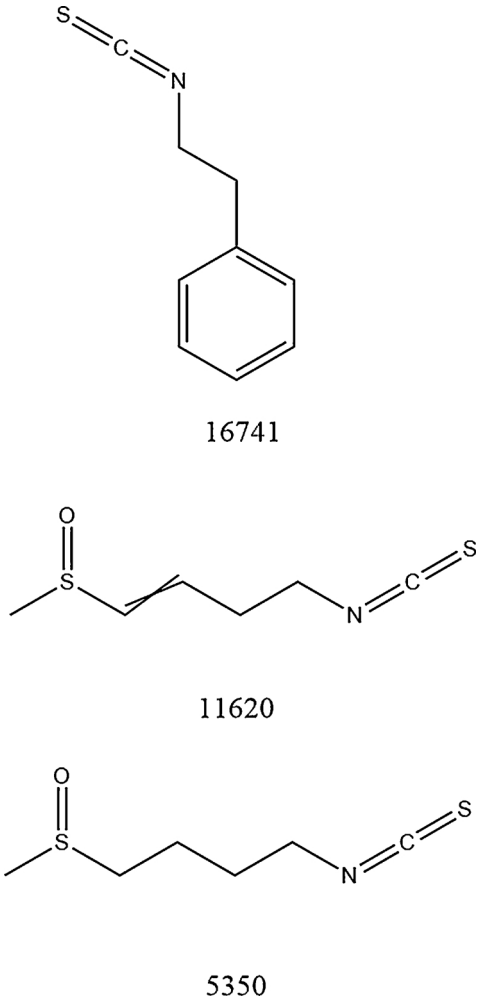

Fig. 1. Structures of the SFN and its derivatives referred in the main text. SFN (CID: 5350), sulforaphene (SFE; CID: 11620), and phenethyl isothiocyanate (PEITC; CID: 16741).

Very recently, *in vitro* and *in vivo* studies have shown SFN is effective in treating different stages of breast cancer. The following characteristics of SFN are effective in treating and arresting breast cancer development: (1) anti-angiogenic; (2) apoptotic; (3) inhibiting cell cycle; (4) chromatin remodeling (5) inhibiting P-450-mediated drug metabolism (phase 1 metabolism); (6) inhibiting drug conjugation (phase 2 metabolism). The compound is also effective in treating triple-negative breast cancer (TNBC) [ER$^-$PR$^-$Her2$^-$], the most incurable type of breast cancer [7–9]. As mentioned above, SFN has a variety of effects on breast cancer cells (based on *in vitro* studies). However, there are hardly any clinical trials to investigate effects of SFN or cruciferous plant extracts against breast cancer (Table 2).

A clinical trial study (NCT00843167) studied chemopreventive effect of SFN on several biomarkers. It has been shown there is no significant decrease in histone deacetylase (HDAC)3, HDAC6, H3K9, H3K18 and p21 activities between placebo- and SFN-treated interventional groups [10,11]. However, SFN inhibits HDAC6-mediated PTEN activation in TNBC nude mice bearing MDA-MB-231 xenografts [7]. In addition, SFN has been studied in combination with anticancer drugs [4,8,12,13]. Almost 10–21% of patients suffering from breast cancer who received doxorubicin (DOX) show compromised cardiac function [14]. Therefore, DOX has been previously suggested to be administered in combination with radiotherapy to reduce its adverse effects [15]. An adjuvant therapy of SFN also reduced DOX-related cardiomyopathy [13,16]. A phase II clinical trial (posted on ClinicalTrials.gov in May 2019) studied the effects of SFN on DOX-related cardiomyopathy breast cancer patients (NCT03934905). The SFN-containing broccoli sprout extracts combinatorial treatment has been also designed to improve efficacy and safety of breast cancer radiotherapy (NCT00894712). Patients who took a third generation aromatase inhibitors, tamoxifen or fulvestrant and who have a documented evidence of progressive disease after six months, have shown secondary resistance [17]. SFX-01, a therapeutic synthetic SFN, has been used in combination with aromatase inhibitors, tamoxifen and fulvestrant to study their effects on drug resistance. However, though this clinical trial has been completed, its results have not yet been reported (clinicaltrials.gov identifier: NCT02970682).

Despite these clinical trials, the efficacies of SFN on its target molecular regulators remain unclear. The real targets of SFN remain vague, especially those of signaling pathways involved in different stages of breast cancer. A number of studies used SFN in combination with other drugs to overcome resistance or adverse effects of current chemotherapy as well as improving SFN efficacy in breast cancer. Therefore, a review of this topic is vital and which will highlight research gaps and raise relevant questions to assist future research on SFN and its efficacy in treating breast cancer. This review surveyed literature on the subject of molecular properties and anticancer effects of SFN. The results showed there is a dearth of publication on SFN and its effect on breast cancer treatment.

2. Anti-oxidant activities of SFN

Oxidative stress and reactive oxygen species (ROS) damage the DNA via epigenetic changes, and they reduce expression of anti-oxidants of superoxide dismutase (SOD) family, cause genomic instability, activate nuclear factor-κB (NF-κB) transcription factor and inflammatory

Table 2
Clinical trials completed or started on SFN and breast cancer.

NCT Number[a]	Title	Status	Conditions	Participants
NCT00982319	Study to Evaluate the Effect of Sulforaphane in Broccoli Sprout Extract on Breast Tissue	Completed	Breast Cancer	34
NCT00894712	Topical Application of Sulforaphane-containing Broccoli Sprout Extracts on Radiation Dermatitis	Completed	Breast Cancer Dermatitis	12
NCT03934905	Protective Effects of the Nutritional Supplement Sulforaphane on Doxorubicin-Associated Cardiac Dysfunction	Not yet recruiting	Anthracycline Related Cardiotoxicity in Breast Cancer	70
NCT00843167	Broccoli Sprout Extract in Treating Women Who Have Had a Mammogram and Breast Biopsy	Completed	Breast Cancer Precancerous Condition	54
NCT02970682	SFX-01 in the Treatment and Evaluation of Metastatic Breast Cancer	Completed	Breast Neoplasm	60

[a] Clinicaltrials.gov identifier.

signaling cascade, and cause inflammation leading to cancer [18–20]. It can be concluded anti-oxidants prevent cancer development [21].

Cruciferous vegetables containing SFN have anti-oxidant properties [22]. The SFN is derived from GFN and the latter is a member of the GLS family that is hydrolyzed by endogenous myrosinase and makes active ITCs, such as SFN [23]. Myrosinase is located in a different cellular apparatus and kept apart from GLSs. This enzyme is temperature-sensitive and can be inactivated when exposed to a high temperature [24].

The anti-oxidant activity of SFN has been investigated on MCF-7, MDA-MB-231, and SK-BR-3 breast cancer cell lines. Several studies found that SFN in combination with taxanes or paclitaxel inhibits proliferation of MCF-10A, MDA-MB-231, MCF-7, SUM149, and SUM159 breast cancer cell lines by controlling the transcription factor NF-κB [25,26]. Another study showed consuming broccoli sprouts for a week reduces oxidative stress and improves cholesterol metabolism. The ROS production was 40% lower in SFN group than the Control, and lipid peroxidation was 63% higher in the H_2O_2 group. It has been shown that SOD activity is much higher in cells incubated with 24 h of SFN [27]. The activity of Peroxisome proliferator-activated receptor-gamma co-activator (PGC)-1α, which plays a central role in the regulation of cellular energy metabolism, is increased by 69% after 24-h treatment with SFN [27,28]. However, nuclear factor (erythroid-derived 2)-like 2 (Nrf2) protein expression is reduced by 17% in the 24 -h group compared with the 1 h groups [27].

SFN suppresses cytochrome P450 enzymes and activates phase II enzymes via Nrf2 transcription factor, and induction of tissue glutathione (GSH) levels [29,30]. The effect of SFN on CYPs (1A1, 1A2, 1B1) involved in estrogen catabolism depends on breast cell line. Cell lines showed a reduction in CYP1A1 protein levels. Specifically, an increased level of CYP1A2 and a decreased level of CYP1B1 expression were found in MCF-10A. This suggests the natural compound L isomer of SFN affect expression of P450 s involved in estrogen metabolism [31]. In addition, SFN and its derivatives inhibit CYP1A1 and CYP1A2 enzyme activity induced in MCF-7 breast cancer cells [32]. The SFN detoxifies carcinogenic compounds by activating phase 2 enzymes. SFN R-enantiomer increases hepatic glutathione S-transferase and quinone reductase whereas the S-enantiomer has no effect. The R-enantiomer is more effective in up-regulating GSTα, GSTμ and quinone reductase protein levels [33,34].

Anti-oxidant response elements (AREs) in the 5′-flanking region of the CYP2A6 gene have been identified. Electrophoretic mobility shift assays have demonstrated that Nrf2 bound only to ARE1 [35]. The Nrf2 protects cells against various toxic compounds (Fig. 2). It also reduces response to chemotherapy agents. Thus, cancer cells with a high level of ARE activity show resistance to chemotherapy [36]. The Nrf2 inhibitors sensitize cancer cells to chemotherapy [37]. It is suggested Kelch-like ECH-associated protein 1 (Keap1)-Nrf2 system is a potential therapeutic target against cancer. Keap1 is a protein bound to Nrf2 and causes Nrf2-polyubiquitination and in turn, directs Nrf2 to proteasome-mediated degradation. Keap1 inhibitors, therefore, prevent Nrf2 degradation and increase Nrf2 level [38]. In order to activate Nrf2, sensor cysteines in Keap1 should be chemically modified, of which C151, C273 and C288 are crucial [39]. Small molecules interact with KEAP1 cysteine residue 151 (C151) are able to activate NRF2 [40]. Studies have shown SFN is able to activate Nrf2 which regulates CYP2A6 [27,35]. It has been shown SFN activates upstream E1b promoter transcription in human lung and liver cells, but not in breast cancer. Based on genetic data, two major DNase I hypersensitive regions (HS-1 and HS-2) have been identified to intervene sequence separating E1b from the downstream E1 promoter. It has been shown SFN specifically activates HS-2 through an anti-oxidant response element (ARE) [41].

Breast cancer resistant protein (BCRP), an ATP-binding cassette transporter protein also called as ABCG2, induces resistance to chemotherapy in breast tumors. The activity of BCRP depends on aryl hydrocarbon receptor (AHR). Although SFN plays its role as agonist of AHR which positively regulates Nrf2 signaling, it does not affect BCRP expression [42]. Although there are reports regarding the activity of SFN on Nrf2, Keap1, and AHR, there is no documented target for SFN proving its specific target in Nrf2 pathway. This though needs further research. In general, the effect of SFN is dose-dependent. It responds to oxidative stress at low doses and causes apoptosis at high doses playing its dual role as an anti-oxidant and an antitumor [27].

3. The effects of SFN on BRCA-deficient breast cancer cells

Prevalence of BRCA1/2 mutations (10.6%) has been observed in patients with TNBC who did not have a significant family history of it [43]. BRCA proteins are involved in DNA repair in proliferative cells whose mutations lead to Myc overactivation which is correlated with increased expression of the homologous DNA recombination enzyme RAD51 in BRCA-mutants/sporadic TNBC patients [44]. Overactivation of Myc and RAD51 has been observed in strongly metastatic breast cancer patients, including TNBC patients. In metastatic breast cancer, RAD51 expression is increased. In an experiment on mice, it was found reduction in RAD51 expression has a reverse association with metastasis. In short, the expression of RAD51 changes metastatic gene expression profile of cancer cells [45]. The BRCA-mutated TNBC cells are also resistant to PARP inhibitors [44]. Accordingly, mutations in BRCAs not only promote breast cancer progression and metastasis through Myc oncogenic overactivity, it (lack of BRCAs) also sensitizes breast epithelial cells to ROS levels [46]. The SFN and resveratrol (RSV), a phytoestrogen, are known to reverse multidrug resistance in cancer cells. Their efficacy is boosted when they are used in combination with approved cancer drugs. This has the outcome of sensitizing cancer cells to standard chemotherapeutic agents [47]. The BRCA1 deficient-cells in particular, have great sensitivity to ROS accumulation. Studies have shown combined treatment of SFN with RSV can reduce DNA damage in BRCA1-deficient and -proficient cells. Additionally, efficacy of combined SFN and RSV on preventing oxidative stress/DNA damage in mammary epithelial cells has been documented [46]. Literature has also suggested the effectiveness of these compounds on the combined

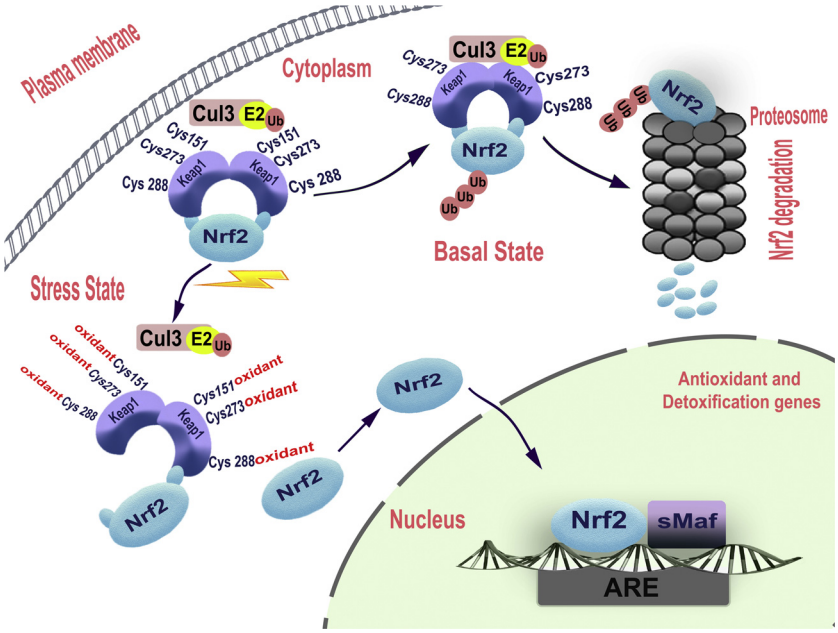

Fig. 2. Nrf2 mechanism of detoxification in breast cancer cells. Nrf2 protects breast cells from oxidant agents. It expresses several genes related to detoxification including antioxidant genes. Therefore, higher activity of Nrf2 in breast cancer cells increases chemoresistance. In order to sensitize breast cancer cells to chemotherapy, Nrf2 should be suppressed. Keap1 is an endogenous inhibitor of Nrf2. It has three key positions for Cysteine residue (Cys151, Cys273, and Cys288) which activate Keap1 with their oxidant states and directs Nrf2 to proteasome for degradation. However, to protect breast cells from cellular damage as a result of oxidants agents, Nrf2 should be activated. Although a few experiments showed SFN could increase Nrf2 activation, Nrf2-based antioxidant mechanism of SFN is still not clear.

responses of BRCA1 and Nrf2 to oxidative stresses.

In contrast to AHR which positively regulates Nrf2 signaling, ERα inhibits Nrf2 signaling in ER positive breast cancer cells. The ROS play an important role in Epithelial-mesenchymal transition (EMT). NADPH-dependent oxidoreductase 1 (NQO1) and heme oxygenase I (HMOX1) are two key enzymes with anti-oxidant activity in which their mRNA levels are increased by SFN treatment [48]. TGF-β-induced EMT in the MCF7 cells is also reduced by HMOX1 [49]. Combinatorial treatment of SFN with 3,3′-diindolylmethane (DIM), 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) and 17β-estradiol (E2) in MCF-7 breast cancer cells showed that co-treatment with E2 significantly downregulated NQO1 and HMOX1 [48]. NQO1 and HMOX1 reductions are associated with increased ERα. However, different combinations of DIM and SFN or TCDD and SFN induced NQO1 and HMOX1 mRNA expression to higher levels compared with using SFN alone which was related to higher activity of AHR [50]. Exemestane, which is a synthetic steroidal inhibitor of aromatase reaction and usable for ER positive breast cancer, could also upregulate NQO1 and HMOX1 leading to suppression of inflammation as well as ER activity. It has been shown that SFN in combination with exemestane synergistically increases the role of exemestane against ER positive breast cancer [51]. Comparing these results with those of earlier ones about the effect of SFN on BRCA1-deficient TNBC cells shows E2 reduces the efficacy of SFN in ER positive breast cells, and SFN in combination with tamoxifen provides better results. Accordingly, SFN either alone or in combination with other anti-oxidant agents is suggested for ER negative breast cancer cells which do not respond to hormone therapy.

4. The apoptotic effects of SFN on breast cancer cells

Apoptosis has different signaling pathways including ROS-dependent apoptosis, Fas-dependent apoptosis, p53-dependent apoptosis and p53-independent apoptosis [21]. Caspases are the most important enzyme family involved in apoptosis (Fig. 3). Non-steroidal anti-inflammatory drugs (NSAIDs), such as ibuprofen, naproxen, and ketorolac, inhibit both categories of cyclooxygenase (COX) and caspase enzymes at physiologic concentrations [52]. Apoptosis is under negative control of *Baculovirus inhibitor of apoptosis repeat containing* (*BIRC2/3*) and *X-linked inhibitor of apoptosis* (*XIAP*) genes encoding cellular inhibitor of apoptosis proteins 1/2 (cIAP1/2) and XIAP respectively [53,54]. In addition, high expression of *cIAPs* and *XIAP* has been reported in breast cancer patients [55]. Meanwhile, it has been shown that apoptosis can be induced by phytochemicals through *cIAPs* downregulation in breast cancer cells [21,56].

Many experiments have confirmed the apoptotic effect of SFN on breast cancer cells which results in DNA fragmentation in MDA-MB-231, MDA-MB-468, MCF-7 and T-47D breast cancer cells. The SFN triggers apoptosis in MDA-MB-231 by activation of extrinsic pathway of apoptosis from Fas ligand which in turn, leads to activation of Caspase-3, Caspase-8 and poly (ADP-ribose) polymerase (PARP)-1. In contrast, apoptosis in other breast cancer cells is started by activation of intrinsic pathway by decreasing level of BCL-2 expression, increasing of Cytochrome-C and activation of Caspase-3 and Caspase-9, instead of caspase-8 [57]. In addition to caspases, PARP-1 is normally involved in the routine repair of DNA damage and adds poly (ADP ribose) polymers in response to a variety of cellular stresses which activates apoptosis by the proteolytic action of suicidal proteases, such as caspases, calpains, cathepsins, granzymes and matrix metalloproteinases (MMPs) [58]. It has also been reported 30μM of SFN could reduce MDA-MB-231 cell growth and by caspase-3 activation-induced apoptosis. However, autophagy protect the cells from SFN-induced apoptosis and use of autophagy inhibitors, such as bafilomycin A1, which increases the apoptotic effect of SFN noticeably via increase of BAX, caspase-3 cleavage, and PARP-1, and decrease of mitochondrial membrane potential [59].

In addition, nanoparticles significantly improve apoptotic effects of SFN. Using gold-coated iron oxide nanoparticles loaded by L-SFN, the biologically active form of SFN show apoptotic effect on MCF-7 breast cancer cells. In fact, it has been documented unloaded nanoparticles have little cytotoxicity. Moreover, SFN loaded nanoparticles reduce gene expression of anti-apoptotic genes, such as BCL-2 and BCL-XL in MCF-7 breast cancer cells [60]. In order to improve bioavailability and efficiency of SFN, its co-delivery in combination with curcumin has been suggested. PEGylated gold coated Fe3O4 magnetic nanoparticles have been used as delivery system of SFN and curcumin to MCF-7 cells and which show an increase in the rate of apoptotic and necrotic deaths, and inhibit the ability of migration in MCF-7 cells [61]. Toxicity of encapsulated SFN with monomethoxypoly (ethylene glycol)-poly (ε-caprolactone) (mPEG-PCL) has been evaluated. The mPEG-PCL micelle shows little cytotoxicity in MCF-7 cell line with concentration up to 1.5 mg/ml, whereas the SFN-loaded mPEG-PCL micelles at all concentrations are found to be cytotoxic in the case of MCF-7 cell line. The SFN encapsulated with mPEG-PCL micelles efficiently triggers apoptosis

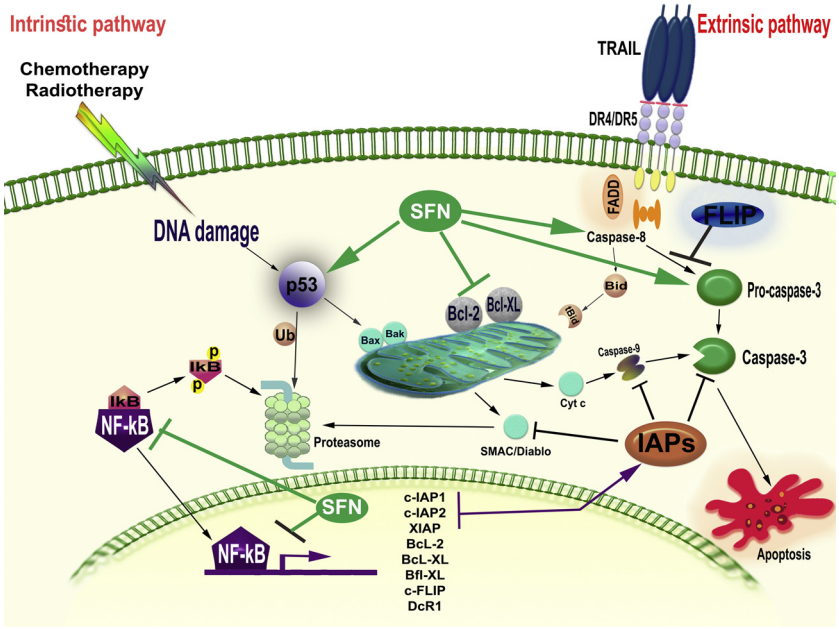

Fig. 3. The mechanism of apoptosis in breast cancer cells. NF-κB is a nuclear factor which expresses inhibitors of apoptosis including caspase inhibitors (c-IAP1/2 and XIAP), stabilizers of mitochondria including Bcl-2, Bcl-XL and Bfl-XL, death domain (FADD) inhibitors such as c-FLIP and inhibitor of death receptors DR5/DR5 such as DcR1. NF-κB, itself, is inhibited by IκB. Apoptosis has two mechanisms of actions: (1) Extrinsic pathway which requires the activation of death receptors by exogenous agents, such as chemicals or immune cells presenting death ligands like TRAIL and (2) Intrinsic pathway started by DNA damage or oxidant agents. Extrinsic pathway activates caspase 8 whereas intrinsic pathway is triggered by tumor suppressor, p53, which then activates caspase 9. Both apoptotic pathways affect mitochondrial membrane and release Cyt c from mitochondria, and which then activate caspase 3 eventually leading to apoptosis. In order to activate caspases, c-IAPs and XIAP should be suppressed and instead, SMAC/Diablo should be activated. The SFN activates extrinsic pathway through activation of caspase 3 and caspase 8. The SFN and other ITC can reduce the Bcl-2 and Bcl-XL at the transcriptional level. The effects of SFN on different level of apoptotic pathways have been shown here.

in MCF-7 cells [62].

In other studies, SFN and other ITCs have been used in combination with other compounds. For instance, SFN in combination with clofarabine produce marked improvement by increasing apoptosis in non-invasive stage of breast cancer. In fact, 10μM concentration of SFN show effects on DNA methylation, increases the expression of silenced tumor suppressors, PTEN and RARβ2, via hypomethylation as well as the overexpression of DNA methylation regulators, such as DNMT1 and tumor suppressor proteins, p53 and p21 in MCF-7 and MDA-MB-231 breast cancer cells [63]. Apoptosis is, then, activated in a p53-dependent manner [21]. Further, apoptosis is, directly or indirectly, related to cyclin dependent kinases involved in cell cycle progression or tumor suppressors, such as p53 and p21.

Early growth response 1 (Egr1) regulates the expression of p15-CDKN2B and p21-CDKN1A required for cell cycle arrest and apoptosis induction [64]. Erg1 also triggers apoptosis through upregulation of PTEN and nuclear factor κB (NFκB) [65]. Antitumor effects of SFE, a SFN derivative (4-methylsufinyl-3-butenyl ITC; PubChem CID: 11620), is facilitated by Egr1 in TNBC (Fig. 4). It has been shown that SFE is a potential anti-TNBC compound whose effects are mediated by Egr1, a tumor suppressor [66]. On the other hand, combinatorial treatment of SFN and 5-Flurouracil shows noticeable decrease in number of MDA-MB-231 cells, a TNBC cell line, by inducing apoptosis and premature senescence [67]. Paclitaxel is another plant-based anticancer agent that targets microtubule function, suppresses cell cycle, and induces apoptosis [68]. It has been shown combinatorial treatment of SFN and paclitaxel increases the rate of paclitaxel-induced apoptosis in MDA-MB-231 and MCF-7. The SFN in combination with paclitaxel increases the activity of caspase-3, caspase-8, and caspase-9, and decreased NFκB signaling pathway and bcl-2 expression in breast cancer cells [25]. However, knocking down caspase-9 and treating with caspase-9 inhibitor in combination with SFN sensitize MCF-7 cells to SFN-induced apoptosis through caspase-9 independent mechanism [69]. The SFN in combination with gemcitabine increases in gemcitabine efficiency in breast cancer. The SFN also shows cytotoxic effect on MCF-7 cells by triggering apoptosis. The SFN also shows apoptotic and anti-

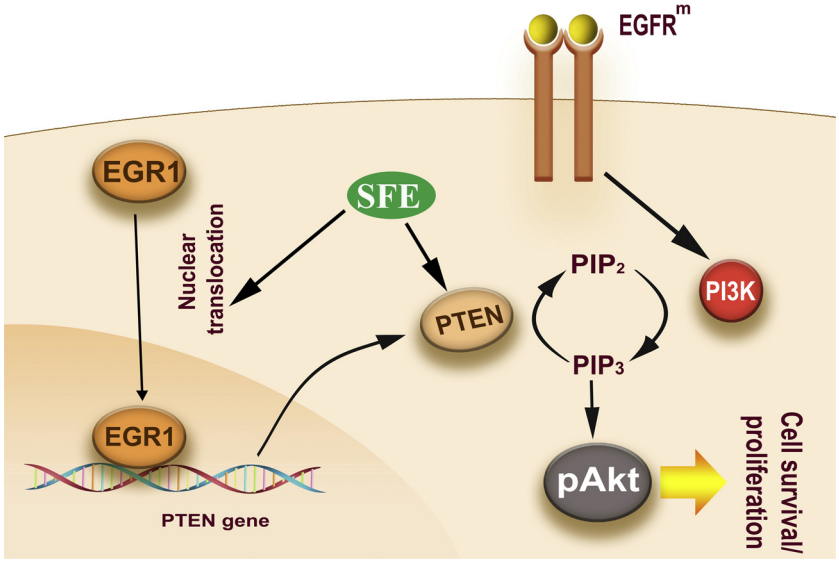

Fig. 4. The mechanism of ERG1 in breast cancer. ERG1 play its role as tumor suppressor by expression of PTEN. The activation of PTEN is necessary for inhibition of PI3K/Akt pathway. Sulforaphene (SFE), a SFN derivative, has been shown to reduce TNBC proliferation by mediating ERG1/PTEN axis.

inflammatory effects by downregulation of Bcl-2 and COX-2 [70].

Mitochondrial ROS (mitoROS) is very important for cellular homeostasis but its excessive production as well as excessive calcium ions can enter mitochondria leading to cell injury and death. The Bcl-2 plays its role as regulator and sensor of mitoROS and Bcl-2 deactivation affects mitochondria function and leads to cytochrome C release from mitochondrial inner membrane to cytoplasm and start apoptotic death in a process called intrinsic apoptosis [71]. In contrast to Bcl-2 which should be downregulated in breast cancer, caspase-3 along with cytochrome C and apoptotic protease activating factor-1 (APAF-1) are intrinsically apoptotic proteins whose upregulation results in intrinsic apoptotic cell death [72].

As mentioned earlier, SFN in combination with many anticancer compounds inhibits Bcl-2. It activates intrinsic apoptosis through overexpression of apoptotic key proteins, caspase-3 and Bax. Adaptor proteins growth factor receptor-bound protein 2 (Grb2) and p66Shc play an important role in activation of downstream EGFR signaling [73]. Many cancer patients have increased levels of p66Shc despite it being a negative regulator of proliferation; p66Shc does this by preventing Grb-2 from binding and activating Ras which then downregulates activities of MAP kinases. It has to be noted p66Shc isoform of ShcA is upregulated in metastatic human breast cancer cell line MDA-MB-231 [74]. Controversially, p66Shc has an opposite function and triggers ROS-dependent apoptosis because it has a cytochrome C–binding region that is responsible for its interaction with cytochrome C. p66Shc reduces equivalents of mitochondrial electron transfer chain through oxidation of cytochrome C resulted in generating mitoROS [75]. It has been shown immortalized mouse embryonic fibroblasts (MEF) lacking p66Shc are more resistant to SFN-induced apoptosis compared with wild type MEF. This points to a critical role for adapter protein p66Shc in SFN-induced apoptosis. Non-tumorigenic mammary epithelial cell line, MCF-10A, i also found to resist SFN-induced ROS production and apoptosis [76]. It has also been shown activation of protein kinase C involved in gene expression, protein secretion, cell proliferation, and the inflammatory responses is necessary for ROS-induced apoptosis [77,78].

Treatment with SFN also increases Ser36 phosphorylation of p66Shc and its mitochondrial translocation in MCF-7 and MDA-MB-231 [76]. As Her2 is another key tyrosine kinase of breast cancer cells, such as SK-BR-3 and MCF-7 cell lines, treatment with lapatinib, a tyrosine kinase inhibitor that specifically targets Her2, in combination with one of ITCs (e.g., SFN and erucin), induces apoptosis more effectively in MCF-7 cells compared with using either of these agents alone [79]. In general, tumorigenic cells are significantly susceptible to SFN and activation of EGFR signaling and its adapter, p66Shc. Additionally, PKCB signaling is required for SFN-induced apoptosis in breast cancer cells, in both $Her2^+$ and TNBC cells. However, both nontumorigenic cells and normal cells are resistant to ROS-dependent (caspase-9 independent) SFN-induced apoptosis.

Studies discussed above suggest SFN affects $Her2^+$ and $Her2^-$ breast cancer cells. They show how SFN targets enzymes which are common in both categories of breast cancer cells. Estrogen receptor (ER) positive cancer cells respond to selective estrogen receptor modulators (SERMs), such as tamoxifen and afimoxifene (4-hydroxytamoxifen) [80]. The effects of ITCs, such as SFN and erucin in combination with 4-hydroxytamoxifen, have been studied in ER^+ breast cancer cells, T-47D, MCF-7 and BT-474. The SFN reduces Bcl-2/Bax ratio and level of survivin, and in contrast, increases level of PARP cleavage. Interestingly, ITCs also sensitize 4-hydroxytamoxifen-resistant T-47D and MCF-7 cells to 4-hydroxytamoxifen [81]. Previous studies have clearly shown SFN in combination with other anticancer agents is very effective in all kinds of breast cancer. Withaferin A (WA) a steroidal lactone derived from *Acnistus arborescens*, *Withania somnifera* and other members of Solanaceae family, has been previously reported as antineoplastic agent against breast cancer [82]. It has also been shown SFN in combination with WA inhibits MCF-7 and MDA-MB-231 breast cancer cell activity and it also negatively affects Bcl-2. Additionally, SFN raises the BAX activity leading to apoptosis [83].

5. The effects of SFN on cell cycle

The SFN is able to target mitosis through inactivation of tubulin polymerization. In order to show immediate effects of SFN on cell division, BALB/c mouse mammary carcinoma cells, F3II cells, were treated with 15 μM concentration of SFN which showed a blockage at the earlier phases of M (prophase/prometaphase) indicating inhibitory effects of SFN on mitotic spindles and tubulin polymerization. Furthermore, daily injection of SFN for 13 days in these mice showed significant reduction in tumor masses compared with the control. It has also been shown SFN could reduce DNA synthesis by its effect on PCNA [84] and it also affects transcription factors involved in gene regulation of cell cycle checkpoints. Cell cycle checkpoints are under control of tumor suppressor proteins. *SERTA domain containing 1* (*SERTAD1*) is an E2F-responsive promoter which stimulates E2F1 and DP1 transcriptional activity while also being responsible for cyclin D1/CDK4 which is resistant to CDK4/6 inhibitors, CDKN2A. Therefore, *SERTAD1* plays its role as inhibitor of G1 tumor suppressors. Although the inhibitory role of SFN against G2/M has been previously observed in MDA-MB-231, MDA-MB-468, MCF-7, and T-47D breast cancer cells [57], it has recently been shown SFN downregulates *SERTAD1* in ZR-75-1 breast ductal carcinoma cells leading to cell cycle arrest in G1/S phase. Furthermore, SFN could also downregulate cyclin D2 and HDAC3 which suggests SFN not only prevents breast cancer, but it also exerts antitumor activities in established breast cancer cells [85] (Fig. 5).

The HDAC inhibitors are known to suppress cancer stem cell (CSC) population in multiple types of cancer cells including breast cancers. It has been also shown that HDAC8 and HDAC3 associated with PI3K/Akt pathway regulate CSCs in TNBC cells. The detection of $CD44^+/CD24^-/CD49f^+$ as CSC markers indicates HDAC inhibitors suppresses CSC subpopulation of TNBC cells through β-catenin downregulation *in vitro* and *in vivo* studies [86,87]. The SFN inhibits HDAC activity and decreases expression of ERα, EGFR, and Her2 in MDA-MB-231, MDA-MB-468, MCF-7, and T-47D estrogen receptor positive and negative invasive breast carcinoma. However, the acetylation of H3 or H4 is not observed [57]. In a clinical trial, HDAC3 levels were measured before and after the GFN supplementation providing SFN in benign, ductal carcinoma *in situ* (DCIS), or invasive ductal carcinoma (IDC) breast

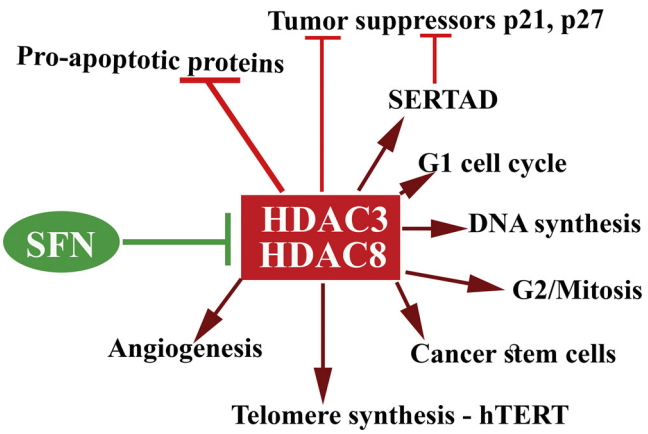

Fig. 5. The effects of SFN on oncogenic functions of breast cancer cells through inhibition of HDACs. SFN, alone or in combination with other agents, reduce DNA stability and cell division of breast cancer cells by affecting Cyclin D1/D2, CDK4/6 (G1 CDKs), cell cycle S phase (PCNA), tumor suppressor inhibitors (SERTAD), angiogenesis (VEGFR), and telomere synthesis (hTERT). Caspase 3 and 8 are also increased by SFN. HDACs, and most frequently HDACs 3 and 8, control gene expression and chromatin functionality of all kinds of cancer including breast cancer.

tissues [11].

Genistein (GEN) is a compound extracted from soy inhibits DNA methyltransferases (DNMT) and when combined with SFN, which is known as HDAC inhibitor, suppresses cell cycle in G1 and G2 phase in MCF-7 and MDA-MB-231 breast cancer cells respectively. The SFN/GEN combinatorial treatment also downregulates HDAC2 and HDAC3 as well as Kruppel-like factor 4 (KLF4) and telomerase reverse transcriptase (hTERT) which play an important role in stem cell formation. Although SFN significantly reduces HDAC3 levels in peripheral blood mononuclear cell (PBMC) of patients suffering from DCIS and IDC breast tumors, it is not suggested for a long-term treatment [11]. However, SFN in combination with GEN has been strongly recommended as a more effective treatment in preventing or treating breast cancers by extending tumor latency and reducing tumor volume/size than either of these dietary components administered alone [12]. Another DNMT inhibitor, WA, in combination with SFN synergistically suppresses cell cycle progression from S to G2 in MDA-MB-231 and MCF-7 breast cancer [88]. SFN in combination with epigenetic modifiers downregulate the levels of cyclin D1, CDK4, and pRB. However, the levels of E2F mRNA and tumor suppressor p21 are increased in a p53 independent manner [12,88].

Dose dependent effect of SFN alone or in combination with other agents suppress cell cycle via inhibition of HDACs as well as the reduction of cyclin and cyclin dependent kinases involved in cell cycle, such as cyclin B1, cyclin A, cyclin D2, CDK4/6 and CDC2 [57,88]. Combination of PEITC and SFN in MCF-7 and normal human epithelial breast cells (HME), both as estrogen dependent breast cancer cells, also overexpressed ER related genes which reduced treatment concentration to 0.3μM. Furthermore, the level of p21 and p27 is significantly increased with 0.3μM of SFN concentration [89]. The SFN increases the level of tumor suppressors, such as p21WAF1, p27KIP1, pRB, and CDKN2A, in MCF-7, MDA-MB-231, and SK-BR-3 breast cancer cells but the concentration is 30μM. The combinatorial treatment of SFN with other agents is suggested [59,90].

The ER and Her2 receptors in breast cancer cells normally respond to hormone therapy and anti-Her2 chemotherapy, but TNBC cells do not express ER, Her2, and PR and which means they do not respond to drugs, such as tamoxifen and trastuzumab. Interestingly, the reactivation of ERα gene is evident in treating patients with 20 μg/mL of green tea polyphenols (GTPs) combined with 5 μM of SFN in MDA-MB-231 TNBC cells lacking ER expression. This study confirms this altered gene regulation is related to hypomethylation and hyperacetylation of the promoter of this gene. The MDA-MB-231 breast cell line, which is a tamoxifen resistant cell, is sensitized to treatment with tamoxifen in combination with GTPs and SFN, and cell proliferation is therefore, inhibited. Cell death is significantly increased in MDA-MB-231 cells compared with treatment with tamoxifen alone [91].

6. The role of SFN on angiogenesis and metastasis of breast cancer

The effects of SFN against protein involved in oncogenic signaling in breast cancer cells have not been well-recognized. Basal-like and the TNBC subsets of breast tumors have a poor prognosis and a high potential for metastasis. Overactivation of EGFR has been reported in at least half of basal-like breast cancer (Fig. 6). Mutated EGFR shows oncogenic activity in breast cancer cells [92]. The effects of SFN against EGFR have been studied in other cancer cells including non-small cell lung cancer (NSCLC) cells *in vitro* and *in vivo*. The SFN concentration of 5–20 μM has been shown to decrease cell viability in TKI-resistant (PC9/gef, H1975, A549, CL1-5) and TKI-sensitive (H3255) cells. The IC50 s of SFN are strongly reduced in mutant-EGFRs expressing cells (IC50 = 5.9–14.5 μM) compared with SFN-treated wild-EGFR expressing cells which have the highest IC50 (IC50 = 65 μM). Furthermore, SFN suppresses EGFR phosphorylation in TKI-resistant NSCLC cells. H1975 and PC9/gef cells are highly sensitive to SFN and have shown a blockade of EGFR phosphorylation at 10 μM concentration of SFN [93].

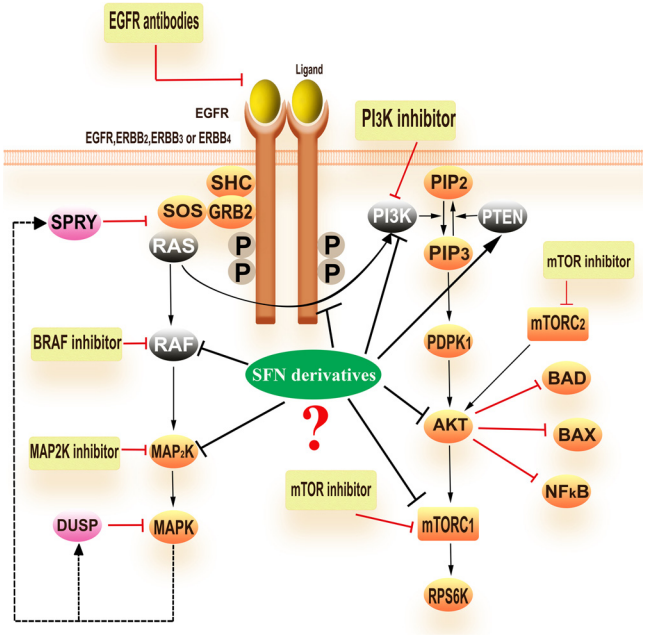

Fig. 6. EGFR and downstream oncogenic signaling in breast cancer. EGFR and its counterpart, Her2 (ERBB2), belong to a group of tyrosine kinase receptors overexpressed in more than half of the breast cancer cases. EGFR leads to activation of two key oncogenic signaling pathways: MAPK and PI3K/Akt pathways. The EGFR is activated by phosphorylation of its kinase domain and other proteins including Shc, Grb2, and SOS are recruited by which RAS triggers kinase cascade leading to the activation of MAPK signaling. The MAPK signaling has several points for inhibition among which Raf is the most important. Some anticancer agents, such as sorafenib and vemurafenib suppress MAP signaling by inhibition of Raf. Some endogenous proteins such as SPRY also regulate MAPK signaling by inhibiting SOS/RAS interactions. The EGFR is able to trigger the parallel PI3K/Akt signaling in which Akt regulates numerous biological actions of breast cells, such as cell proliferation, apoptosis, and autophagy. Akt can directly interact with transcription factors or regulators of apoptosis or activate mTOR complexes which are a key regulator of cellular physiology. mTOR inhibitors have a great role in immunotherapy of cancer. PI3K and Akt can be also inhibited by some chemicals. By controlling MAPK and PI3K/Akt signaling pathways, the cell cycle and metastasis of breast cancer cells are suppressed. Although PI3K/Akt and MAPK pathways are both crucial oncogenic pathways targeted in chemotherapy, the role of SFN and ITCs are not clearly studied in breast cancer and required further investigation. However, the effect of SFN against EGFR has been shown in NSCLC.

However, TKI-sensitive H3255 cell line is not sensitive to SFN as much as TKI-resistant cell lines, suggesting SFN has the potential to treat TKI-resistant cells which are commonly seen among TNBC patients [94].

Vascular endothelial growth factor receptor (VEGFR) is another tyrosine kinase receptor activated by its ligand, VEGF, and it is necessary for angiogenesis and blood vessel formation in many kinds of cancer including breast cancer, colon cancer, and gastric cancer. The VEGF is also linked to poor prognosis in cancer [95]. Hypoxia inducible factor-1α (HIF-1α)-dependent signaling pathway is also involved in cell mobility and promotes metastasis. The effects of SFN on angiogenic pathways have been shown in colon and gastric cancer cell lines, HCT116 and AGS, respectively. It has been shown SFN inhibits expression of HIF-1α in both gastric cancer cell lines whereas it suppresses VEGF expression in HCT116 [96]. It has also been shown SFN could affect metastatic and angiogenic pathways in HT-29 colon cancer cells by inhibition of HIF-1, VEGF, and matrix metallopeptidases (MMPs)-2 and 9 [97]. In HepG2 hepatocellular carcinoma cells, SFN concentration range of 1.25–20 μM suppresses VEGF-A and HIF-1α. It has been reported SFN inhibits biosynthesis of HIF-1α as well [98]. For angiogenesis, a proper communication between endothelial cells (ECs) and pericytes is important. Although VEGFR-2 is elevated, SFN reduces

Table 3
Important targets and mechanisms of Sulforaphane in breast cancer.

Mechanism	Target	Function	Drug/ Combinations	Cell lines	Breast tumor subtypes [137]	Effect of drug	Biological results	References
Chromatin Structure and DNA stability	HDAC1 HDAC2 HDAC3 HDAC4 HDAC6 HDAC7 HDAC8 HDAC9 HDAC10	Histone deacetylation	SFN (alone) SFN + DOX EGCG + SFN EGCG + SFN + TAM SFN + WA SFN +GTPs	MDA-MB-231 MCF-7 MAT B III MDA-MB-157 SK-BR-3 MDA-MB-468 T-47D	TNBC Luminal A (ER^+/PR^+) Adenocarcinoma (Rat) TNBC Adenocarcinoma ($Her2^+$) TNBC Luminal A (ER^+/PR^+)	Decreased	Cell cycle arrest Apoptosis	[11,12,70,83,88,90,91,138,139]
	N6-methyladenosine (m6A)	mRNA modification	SFN (alone)	MCF-7 MDA-MB-231	Luminal A (ER^+/PR^+) TNBC	Decreased	Cell cycle arrest Apoptosis	[90]
	SIN3A	Gene regulation	SFN (alone)	MDA-MB-231 SK-BR-3	TNBC Adenocarcinoma ($Her2^+$)	Downregulated	Cell cycle arrest	[90]
	SAPs (SAP18/30)	Histone deacetylation	SFN (alone)	MDA-MB-231	TNBC	Downregulated	Cell cycle arrest	[90]
	RBBPs (RBBP4/7)	Chromatin remodeling factor	SFN (alone)	MDA-MB-231 SK-BR-3	TNBC Adenocarcinoma ($Her2^+$)	Decreased	Cell cycle arrest	[90]
	MECP2	Transcriptional regulation	SFN (alone)	MDA-MB-231	TNBC	Decreased	Cell cycle arrest	[90]
	MBDs (MBD2/3)	DNA methylation	SFN (alone)	MDA-MB-231 MCF-7 SK-BR-3	TNBC Luminal A (ER^+/PR^+) Adenocarcinoma ($Her2^+$)	Decreased	Cell cycle arrest	[90]
	hTERT	Telomere synthesis	SFN (alone)	MCF-7 MDA-MB-231	Luminal A (ER^+/PR^+) TNBC	Decreased	Cell growth inhibition Apoptosis	[140]
	Cyclin Bs (B1/B2)	Cell cycle (S/G2/M)	SFN + 5-FU	MDA-MB-231 MCF-7 SK-BR-3 SUM149 SUM159	TNBC Luminal A (ER^+/PR^+) Adenocarcinoma ($Her2^+$) TNBC TNBC	Decreased	Premature senescence Cell cycle arrest Apoptosis CSC inhibition	[9,59,90,136]

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Table 3 (*continued*)

Mechanism	Target	Function	Drug/ Combinations	Cell lines	Breast tumor subtypes [137]	Effect of drug	Biological results	References
Cell Cycle and Checkpoints	Cyclin A2	Cell cycle (G2/M)	SFN (alone)	MCF-7 MDA-MB-231	Luminal A (ER^+/PR^+) TNBC	Decreased	Cell cycle arrest Apoptosis	[59,90]
	Cyclin Ds (D1/D3)	Cell cycle (G1/S)	SFN (alone)	MCF-7 MDA-MB-231 SUM159 SK-BR-3	Luminal A (ER^+/PR^+) TNBC TNBC Adenocarcinoma ($Her2^+$)	Decreased	Cell cycle arrest CSC inhibition	[90]
	Cyclin E1	G1/S-specific cyclin E1	SFN (alone)	MCF-7 MDA-MB-231	Luminal A (ER^+/PR^+) TNBC	Decreased	Cell cycle arrest	[90]
	CDK1	Cell cycle	SFN + GTPs	MDA-MB-231	TNBC	Decreased	Cell cycle arrest	[136]
	CDC25C	Cell division	SFN + GTPs	MDA-MB-231	TNBC	Decreased	Cell cycle arrest	[136]
	CDC2	Cell cycle	SFN (alone)	MDA-MB-231	TNBC	Decreased	Cell cycle arrest Apoptosis	[59]
	CDKN1A (p21)	CDK inhibitor PCNA inhibitor	SFN (alone)	SK-BR-3	Adenocarcinoma ($Her2^+$)	Increased	Cell cycle arrest	[90]
	p53	Tumor suppressor	SFN (alone)	MCF-7 MDA-MB-231 SK-BR-3	Luminal A (ER^+/PR^+) TNBC Adenocarcinoma ($Her2^+$)	Increased	Cell cycle arrest	[90]
Apoptosis	BCL-2	Apoptosis regulation	SFN + WA SFN + GEM SFN + PAC	MCF-7 MDA-MB-231 T-47D BT-474 MDA-MB-468	Luminal A (ER^+/PR^+) TNBC Luminal A (ER^+/PR^+) Luminal B ($ER^+/PR^+/Her2^+$) TNBC	Decreased	Apoptosis Cell growth inhibition	[60,62,70]
	Bcl-XL	Apoptosis regulation	SFN (alone) SFN + HTAM	MCF-7	Luminal A (ER^+/PR^+)	Decreased	Apoptosis induction	[60,62]
	BID	Apoptosis regulation	SFN (alone)	MCF-7 MDA-MB-231	Luminal A (ER^+/PR^+) TNBC	Induced	Apoptosis	[141]
	Caspases	Apoptosis regulation	SFN (alone)	MCF-7 MDA-MB-231	Luminal A (ER^+/PR^+) TNBC	Activated	Apoptosis	[141]
Inflammation and Oxidative Stress	CYPs (CYP19/1A1/ 1A2/ 1B1)	Detoxification	SFN (alone)	MCF-7 MDA-MB-231 MCF-10A	Luminal A (ER^+/PR^+) TNBC Non-tumorigenic	Decreased	Decreased de novo estrogen synthesis	[31,32,142,143]
	Keap1	Nrf2 inhibitor	SFN (alone)	MCF-7 MDA-MB-231	Luminal A (ER^+/PR^+) TNBC	Inhibited	Anti-oxidant and anti-inflammatory response	[141,144]
	NF-κB	Inflammations gene expression	SFN (alone) SFN + PAC	MCF-7 MDA-MB-231 SUM149 SUM159 MCF-10A	Luminal A (ER^+/PR^+) TNBC TNBC TNBC Non-tumorigenic	Inhibited	Apoptosis Cell cycle arrest	[9,141,145,146]
	IKKα/β	NF-κB inhibitor	SFN (alone)	MCF-10A	Non-tumorigenic	Decreased	Inhibition of inflammation	[145]

(*continued on next page*)

Table 3 (*continued*)

Mechanism	Target	Function	Drug/ Combinations	Cell lines	Breast tumor subtypes [137]	Effect of drug	Biological results	References
	STAT 1/ 3	Signal transducer and activator of transcription	SFN (alone)	MCF-7 MDA-MB-231	Luminal A (ER^+/PR^+) TNBC	Downregulated	Cell cycle arrest	[141]
Cell Signaling	AKT	Cell proliferation Metastasis Autophagy	SFN + PAC SFN + LAPA	MCF-7 MDA-MB-231 SK-BR-3 MDA-MB-468 BT-474	Luminal A (ER^+/PR^+) TNBC Adenocarcinoma ($Her2^+$) TNBC Luminal B ($ER^+/PR^+/Her2^+$)	Decreased	Cell cycle arrest Apoptosis	[90,147]
	mTOR	Cell growth Cell proliferation Cell motility Cell survival Protein synthesis Autophagy	SFN (alone)	MDA-MB-231 MCF-7 SK-BR-3 MDA-MB-468	TNBC Luminal A (ER^+/PR^+) $Her2^+$ Adenocarcinoma TNBC	inhibited	Inhibited mTOR signaling	[147]
	ERK1/2	Cell proliferation	SFN (alone)	MCF-10A MCF-7	Non-tumorigenic Luminal A (ER^+/PR^+)	Inhibited	Inhibition of inflammation Cell growth inhibition Apoptosis	[145]
Receptors	PDGFR	Tyrosine kinase receptor	SFN	MDA-MB-231	TNBC	Decreased	Inhibition of angiogenesis	[148]
	VEGFR	Tyrosine kinase receptor	SFN	MDA-MB-231	TNBC	Decreased	Inhibition of angiogenesis	[148]
	EGFR	Tyrosine kinase receptor	SFN	MDA-MB-231 MDA-MB-468 MCF-7 T-47D	TNBC TNBC Luminal A (ER^+/PR^+) Luminal A (ER^+/PR^+)	Decreased	Inhibition of cell growth	[57]
	ERα	ER signaling	SFN + DOX	MDA-MB-231 MCF-7 MAT B III ZR-75-1 T-47D	TNBC Luminal A (ER^+/PR^+) Adenocarcinoma (Rat) Luminal A ($ER^+/PR^{+/-}$) Luminal A (ER^+/PR^+)	Decreased	Cell cycle arrest	[16,57,147]
Metastasis and Cell Migration	β-catenin	Regulation and coordination of cell–cell adhesion and gene transcription.	SFN (alone)	MCF-7 SUM159	Luminal A (ER^+/PR^+) TNBC	Decreased	Breast Cancer stem/ progenitor cells inhibition	[149]
	ROCK1	Cell motility Metastasis Angiogenesis	SFN (alone)	MCF-7 MDA-MB-231	Luminal A (ER^+/PR^+) TNBC	Induced	Apoptosis	[141]
	Vimentin	Cytoskeletal component	SFN (alone)	MDA-MB-231	TNBC	Decreased	Inhibition of metastasis	[148]
	MMPs (7/9/14)	Metastasis	SFN (alone)	MDA-MB-231 MCF-7	TNBC Luminal A (ER^+/PR^+)	Decreased	Apoptosis Cell invasion inhibition	[62,148,150]

HDAC: histone deacetylases; DNMT: DNA (cytosine-5)-methyltransferase; **SAP:** Sin3A Associated Protein; **MBD:** Methyl-CpG-binding domain proteins; **hTERT:** human telomerase reverse transcriptase; **STAT:** signal transducer and activator of transcription (STAT) protein family; **mTOR:** Mammalian target of rapamycin; **CDK:** cyclin-dependent kinases; **CDC:** cell division cycle protein; **Bcl:** B cell lymphoma (BCL)- protein family; **BID:** BH3 interacting-domain death agonist; **Cyp:** Cytochrome P450; **NF-κB:** nuclear factor-kappa B ; **IKK:** inhibitor of κB kinase ; **ER:** estrogen receptor ; **PDGFR:** platelet-derived growth factor receptor; **VEGFR:** vascular endothelial growth factor receptor; **EGFR:** epidermal growth factor receptor; **ERK:** extracellular signal–regulated kinases ; **ROCK:** Rho-associated kinase; **MMP:** matrix metalloproteinases; **CSC:** cancer stem cells; **SFN:** sulforaphane; **DOX:** doxorubicin; **PAC:** paclitaxel; **LAPA:** lapatinib; **TAM:** ; **HTAM:** hydroxytamoxifen; **EGCG:** Epigallocatechin-3-gallate; **GEM:** gemcitabine; **WA:** withaferin A; **GTPs:** green tea polyphenols; **5-FU:** 5-fluorouracil.

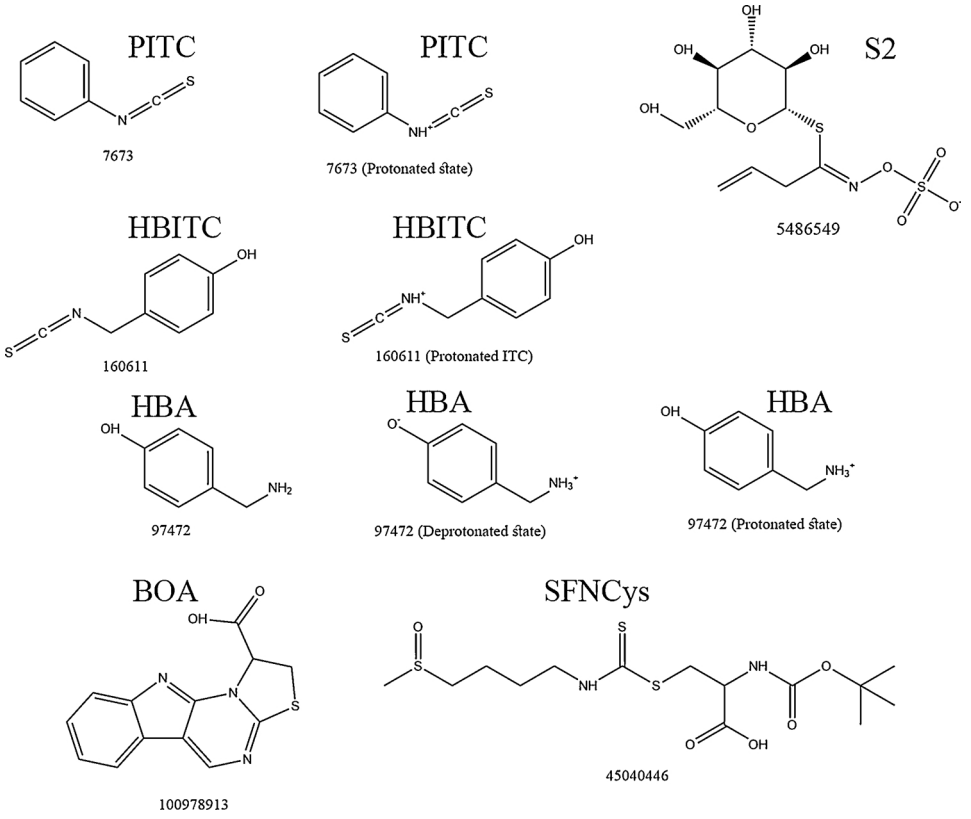

Fig. 7. Structures of the key compounds isolated from *Brassica oleracea* and *Brassica hirta*. The chemical properties of these compounds are shown in Table 1. Plant extract of cruciferous family have been successfully used to treat breast cancer. Other plant derivatives can be also investigated for their effectiveness in treating breast cancer.

VEGF expression in pericytes and accordingly, it is able to influence intracellular communication between ECs and pericytes [99].

MMP-9 (92 kDa type IV collagenase), a biomarker involved in metastasis in different subsets of breast cancer involved in degradation of intercellular matrix, has significantly higher expression in breast cancer cells. The *MMP-9* expression in several breast cancer cell lines including basal-like breast cancer cell lines (e.g. CAL85-1, HCC1395, HCC1143, DU4475, HCC1937, MDA-MB-231 and HCC38) and luminal breast cancer cell lines with HER2 amplification (AU565, UAA-893 and HCC2218) have been shown. Among luminal cell lines, only MCF7 and KPL1 show an increase in *MMP-9* expression [100]. In addition, 12-O-tetradecanoyl phorbol-13-acetate (TPA) stimulates NF-kB and AP-1 DNA binding activity leads to an induction of gene expression including *MMP-9*. TPA-induced *MMP-9* expression has been downregulated by SFN in MCF-7 breast cancer cells. Pre-treatment with SFN also inhibits the binding of TPA-stimulated NF-kB and AP-1 to DNA as well as NF-kB function through suppression of IkB phosphorylation in TPA-treated MCF-7 cells [101]. Anti-angiogenic and anti-metastatic effects of SFN have been studied in selected cell lines of gastrointestinal (GI) cancers and DU145 prostate cancer cells with similar inhibitory effects on HIF-1α and VEGF [102]. However, SFN effects on metastasis and angiogenesis have not been clearly investigated and require further research.

7. SFN affects cancer stem cells

Cancer stem cells (CSCs) are involved in cancer recurrence, and targeting CSCs sensitize patients to therapy [103]. The CSCs are isolated by detection of $CD44^+/CD24^-/CD49f^+$ cells. The effects of SFN have recently been shown on TNBC-inoculated BalbC/nude mice by reducing teratocarcinoma-derived growth factor 1 (TDGF1) expression by administering dose of 50 mg/Kg, and mamosphere CSC formation therefore is inhibited in TNBC cells [86]. The TDGF1 belongs to EGF-CRIPTO/FRL-1/CRYPTIC (CFC) domain and it is connected to TGF-β signaling, which in turn, deactivates anticancer immunity and whereby PI3K signaling promotes cell migration [104]. The SFN also reduces various stem cell markers including Nanog, aldehyde dehydrogenase 1A1 (ALDH1A1), Wnt3, and Notch4 [86]. The SFN treatment has been studied on nasopharyngeal carcinoma (NPC) cells with CSCs-like properties and which shows a decrease in cell population with CSC-related properties (SOX2 and ALDH). Furthermore, SFN causes rehabilitation of Wnt inhibitory factor 1 (WIF 1) expression along with downregulation of DNMT1 in monolayer culture of growing NPC cells, CSCs-boosted NPC tumor spheres and the xenograft nude mice with NPC cells [105].

Moreover, the use of SFN in combination with other agents improves drug cytotoxicity by inhibition of CSC markers. It has been shown sorafenib, a Raf inhibitor, improves the activity of NF-κB which is related to viability and renewal of spheroids. Adding SFN to sorafenib eliminates sorafenib-induced NF-κB binding in pancreatic cancer and then, reduces spheroid formation and ALDH1 activity [106]. The use of taxanes (paclitaxel or docetaxel) against TNBC also causes a higher population of CSCs in TNBC cells through IL-6 upregulation. In contrast to Taxanes, SFN removes CSCs by downregulation of NF-kB p65 and p52. In addition, SFN decreases taxane-induced ALDH and reduces the size and population of primary and secondary mammospheres [9]. The SFN could also promote the effects of other conventional chemotherapy including cisplatin in NSCLC as well as gastric, pancreatic and prostate cancers by inhibition of CSC formation [107–109].

8. Future directions and concluding remark

The current authors searched PubMed database using key words “sulforaphane” AND “cancer” both in titles of publications, and they found 240 articles on anticancer effects of SFN. They then restricted the keywords to “sulforaphane” AND “breast cancer”, and they found only 44 articles specifically written on breast cancer - majority of which were associated with the role of SFN in epigenetic changes of breast cancer cell cycle. There are only few publications on the effects of SFN on signaling molecules including Her2 and ERK as shown in Table 3. Therefore, there is no strong evidence to show SFN has any strong

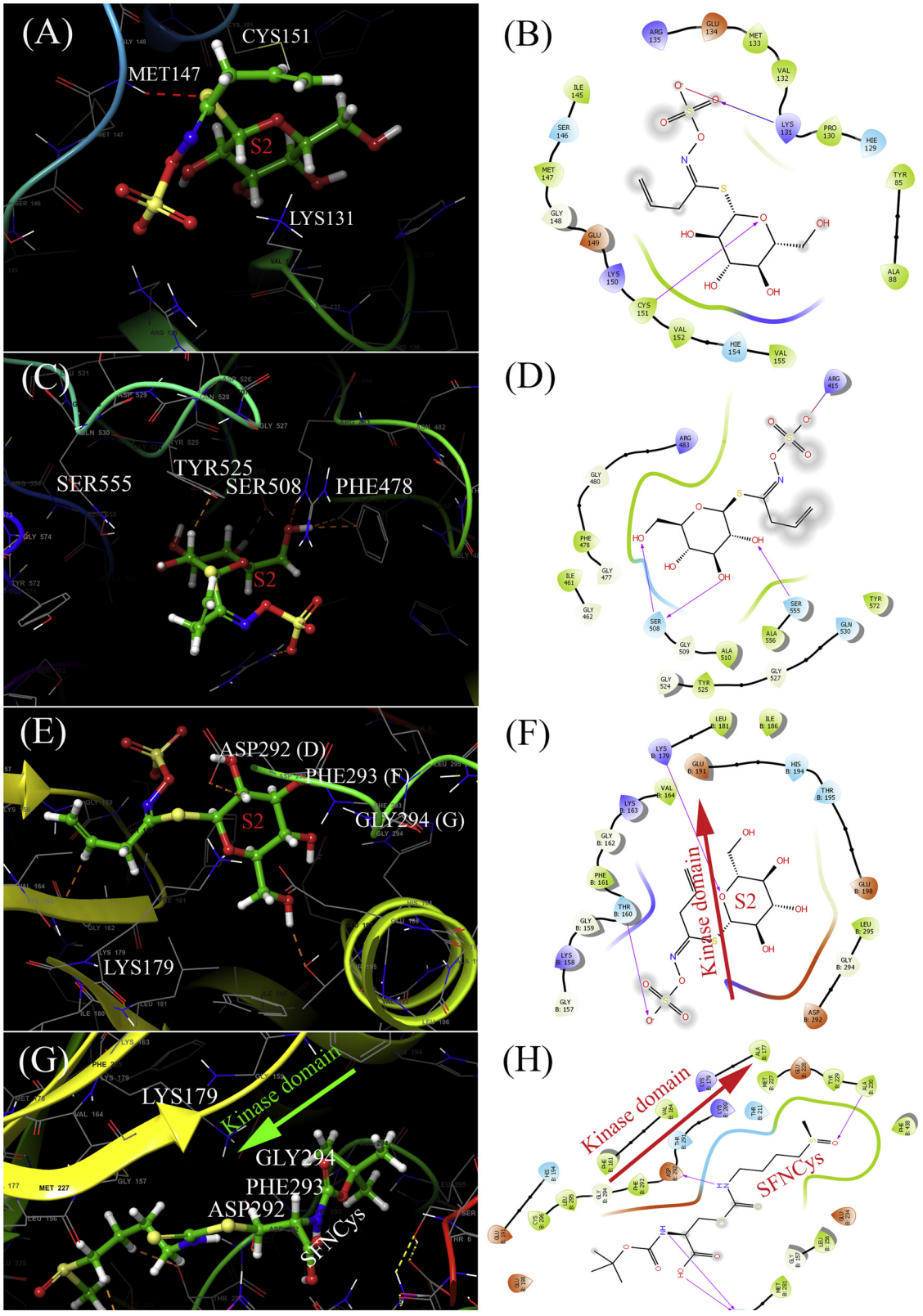

Fig. 8. Interactions of the best cruciferous compounds against Keap1 and Akt. (A–B) S2 against BTB domain of Keap1 (PDB ID: 5DAD). Keap1 inhibits Nrf2 and in turn, downregulates anti-oxidant genes. S2 could successfully bind to BTB domain in which Cys151 plays a key role in sensing oxidative condition of the cells. As BTB has several polar residues including Lys131, Lys150, Tyr85, and Glu149, polar compounds such as S2 are more favorable for binding to this region far better than SFN. **(C–D) S2 against Nrf2 binding site of Keap1 (PDB ID: 6QMC).** This domain recognized by Ser555 is bound to Nrf2 and it redirects towards proteolysis. S2 has a potential to be bound to this domain and it inhibits Keap1/Nrf2 interactions. **(E–H) Akt kinase domain, open position (PDB ID: 3OCB).** Akt kinase domain contains Asp292, Phe293, and Gly294 which are well known as DFG or kinase gate which plays a key role in accepting ATP which it then attaches to Lys179. The current study shows **(E–F)** S2 could enter DFG, occupying the normal position of ATP in kinase activity whereas **(G–H)** SFNCys could not enter the gate and it is bound to the outside of DFG. See Table 1 for information on compounds. S2: 2-Propenyl glucosinolate (S2); SFNCys: D, L-Sulforaphane Boc-L-cysteine.

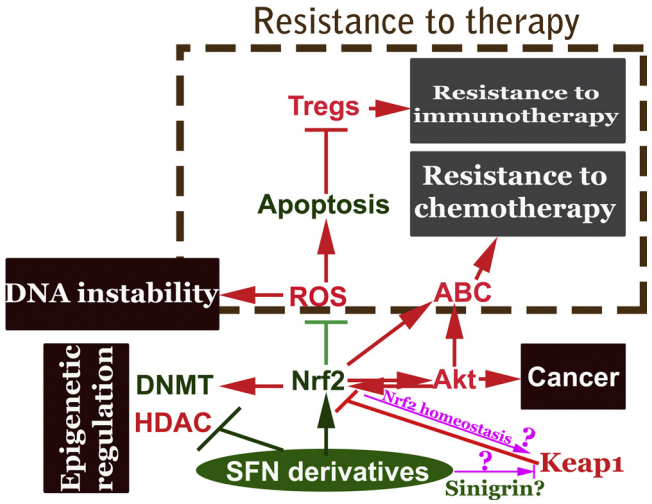

Fig. 9. The contradictory roles of SFN derivatives in breast cancer treatment. The SFN enhances anti-oxidant activity of Nrf2 which may lead to proliferation of regulatory Treg cells which suppress anticancer immunity. On the other hand, Nrf2 upregulates genes related to multidrug resistance transporters (ABC). It also activates Akt, the master regulator of oncogenic signaling pathways. Akt cross talks with other pathways including Nrf2, to promote resistance to chemotherapy. The Nrf2 additionally may regulate its homeostasis by some epigenetic regulations which target Keap1. The SFN derivatives, including sinigrin (a glucosinolate isolated from black mustard) may epigenetically regulate Nrf2 homeostasis by affecting Keap1 (the Nrf2 inhibitor) activation.

action on oncogenic signaling, specifically on MAPK and PI3K/Akt pathways. In sum, future researches need to focus on anti-breast cancer activity of SFN.

This review discussed SFN effects on epigenetic regulation and apoptosis in breast cancer *in vitro* and *in vivo* [88]. Previous studies on SFN and its signaling in different cancer types were reviewed. The authors recommend future studies to focus on the role of SFN in treating breast cancer cells. In addition to SERMs and aromatase inhibitors which are used to treat patients with ER^+ breast cancer, drugs which target the mechanism of tyrosine kinases (Her2, EGFR, and VEGFR) are important depending on the subset of breast cancer [110]. However, TNBC which is the most incurable and aggressive kind of breast cancer does not respond to conventional monotherapy and it requires combinatorial therapy. Although the inhibitory role of SFN on HDACs and apoptotic enzymes has been previously documented in several studies on breast cancer [83], it is important to find out whether SFN possesses multifunctional effects against different signaling of breast cancer. The use of such multifunctional compounds which target different categories of proteins is crucial, especially in case of TNBC which is resistant to conventional monotherapy.

As mentioned, SFN protects DNA structure and breast cells through activation of Nrf2 anti-oxidant signaling. However, interactions and mechanisms of SFN with Nrf2 and its suppressor, Keap1, have not been clearly understood [27,111]. Keap1 has two active sites: a) Nrf2/Keap1 interaction domain (Ser555) leading to proteolysis of Nrf2, and b) BTB domain which contains a few Cys residues and most importantly, Cys151, which is involved in Keap1 activation [112]. In order to activate Nrf2, it is hypothesized SFN may bind to one or both domains and prevents Keap1 from bounding to Nrf2 [113]. As there is no evidence of action of SFN against Keap1, it is important to predict the interaction of SFN and its relatives with Keap1. Using methodology previously described [114], the current authors failed to predict SFN on both domains of Keap1 which had led to a high and unacceptable binding energy. It was interesting to observe that another compound, 2-propenyl glucosinolate, labeled as S2 (CID: 5486549) in Table 1 and Fig. 7, which is isolated from mustard, has the minimum binding energy among examined compounds. The S2 could successfully be bound to both domains, Ser555 and BTB, with binding energies of −7.11 Kcal/mol and −6.87 Kcal/mol respectively. Thus, S2 is predicted to be a better compound against Keap1 compared with SFN but this needs further examination. The molecular interactions of S2 with Keap1 are shown in Fig. 8(A–D). Therefore, this natural compound present in cruciferous extracts can also be considered while investigating anti-oxidant activities of SFN and its relatives. Other compounds shown in Fig. 7 have a higher binding energy. This may be due to the strong polar structure of S2 compared with other compounds including SFN. It has been shown that polycyclic and strongly polar structures reduce the effectiveness of SFN-related compounds against Keap1 [113]. However, SFN has a linear and hydrophobic structure which reduces its biological action depending on the target. Therefore, the current study suggests SFN does not have any effective interactions with Keap1, and this finding is corroborated by an earlier research. The SFN's anti-oxidant activity may be due to its inhibitory effect on proteasomal cysteine deubiquitinases leading to accumulation of Nrf2/Keap1 ubiquitinated complex in cells and which increases intracellular toxic environment. This finally leads to apoptosis and prevents metastatic breast cancer [115,116].

The current study also examined antikinase activity of SFN and the most relevant compounds against Akt, a key modulator of autophagy and cellular vesicular network moderated through mTOR activity (a Akt target) [117]. It is an oncogenic hub leading to metastasis and cell proliferation cross talking with other oncogenic signaling, such as MAPK and Wnt/β-catenin pathways [118]. Akt is a double-edged sword in breast cells; on one hand its activation expresses genes related to detoxification [119], but on the other, its (Akt) deactivation saves breast cells against developing cancer [120]. Using molecular modeling [114], the study found that SFN, which has more positive binding energy, is not predicted to be bound to Akt kinase domain. In contrast, S2 could successfully enter the kinase gate, $DFG_{(292–294)}$ [121], and occupy a position normally filled by ATP, the normal substrate of kinases including Akt and all other kinases (Fig. 8E–H).

Studies have shown Nrf2 has the following effect on breast cancer cells: Anti-oxidant gene expression, and cell growth maintenance [122]. The Nrf2 expresses ATP-binding cassette (ABC) transporters which are key players in drug resistance [123]. Nrf2 activated by SFN can induce P-glycoprotein, ABCB1, ABCC2 (Multidrug resistance-associated protein 2 or MRP2), and ABCG2 [124]. It has been reported recently that PI3K/Akt pathway is associated with ABC transporters in drug-resistant breast cancer cells [125]. The Nrf2 has also been reported to be resistant to immunotherapy [126]. Regulatory T (Treg) cells suppress anticancer immunity and therefore, to combine chemotherapy with immunotherapy, Treg cell proliferation should be inhibited [127]. The Nrf2 pathway is downregulated in Treg cells to sensitize them to apoptosis [128]. As SFN derivatives activate Nrf2 in breast cancer cells, they may also either prevent or promote cancer cell proliferation [129]. Therefore, SFN derivatives may suppress anticancer immunity and may not be used in combination with immunotherapy [126].

Furthermore, it has been clearly shown Nrf2 can induce MDA-MB-231 cell growth by activating DNMT1, DNMT3A, and DNMT3B [130]. On the other hand, Nrf2 activation can be suppressed by inhibition of Akt. Akt/Nrf2 cross talking regulates anti-oxidant and oncogenic effects of Nrf2 on breast cancer cells. In addition, lapatinib activates Nrf2 to reduce ROS level in breast cancer-resistant cells [131]. Estrogen (E2) can also increase Nrf2 activation in E2-dependent MCF-7 breast cancer cells [132]. Therefore, Nrf2 is affected by breast cancer signaling molecules such as Akt and E2. The anti-oxidant effects of SFN derivatives on Nrf2 may be reversed by over activation of Akt in breast cancer cells via the activation of glycolysis [133]. The Nrf2 activation is also inhibited by PI3K inhibitors [132]. In contrast, the inhibition of GSK-3β as a target, which is negatively regulated by PI3K/Akt pathway, upregulates Nrf2 [132]. Thus, Nrf2 activity in breast cancer also depends on the inhibition of p21 and GSK-3β tumor suppressors [134].

According to these contradictory roles of Nrf2, it is believed Nrf2

can self-regulate to maintain its cellular level (Fig. 9). There may be some microRNAs, such as miR-200a, regulated by Nrf2 and which suppresses or activates Keap1, an Nrf2 inhibitor. The inhibition of miR-200a decreases Nrf2 levels while increasing ROS level. In contrast, the overexpression of miR-200a increases the level of Nrf2 to follow anti-oxidant pathway [135].

Literature review has shown a glaring gap in the understanding of mechanisms of Nrf2 homeostasis regulated by SFN in breast cancer. This must be urgently addressed in future research. Biological actions of SFN in breast cancer depend on crosstalking of Nrf2 with Akt which need further elucidations. There is little doubt that SFN and its relatives are highly effective in combination with other anticancer agents [93,103,106,109,136]. Future research needs to elucidate the role of SFN in breast cancer treatment.

Declaration of Competing Interest

The authors do not have any conflict of interests.

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