

# Nrf2-Mediated Antioxidant Response and Drug Efflux Transporters Upregulation as Possible Mechanisms of Resistance in Photodynamic Therapy of Cancers

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**Abstract:** Photodynamic therapy (PDT) is a groundbreaking approach involving the induction of cytotoxic reactive oxygen species (ROS) within tumors through visible light activation of photosensitizers (PS) in the presence of molecular oxygen. This innovative therapy has demonstrated success in treating various cancers. While PDT proves highly effective in most solid tumors, there are indications that certain cancers exhibit resistance, and some initially responsive cancers may develop intrinsic or acquired resistance to PDT. The molecular mechanisms underlying this resistance are not fully understood. Recent evidence suggests that, akin to other traditional cancer treatments, the activation of survival pathways, such as the KEAP1/Nrf2 signaling pathway, is emerging as an important mechanism of post-PDT resistance in many cancers. This article explores the dual role of Nrf2, highlighting evidence linking aberrant Nrf2 expression to treatment resistance across a range of cancers. Additionally, it delves into the specific role of Nrf2 in the context of photodynamic therapy for cancers, emphasizing evidence that suggests Nrf2-mediated upregulation of antioxidant responses and induction of drug efflux transporters are potential mechanisms of resistance to PDT in diverse cancer types. Therefore, understanding the specific role(s) of Nrf2 in PDT resistance may pave the way for the development of more effective cancer treatments using PDT.

**Keywords:** photodynamic therapy, oxidative stress, antioxidant response, tumor resistance, Nrf2

## Introduction

Cancer is presently ranked as the second-leading cause of morbidity and mortality globally; however, it has been predicted that it will become the leading cause of death by the year 2060 because of the increase in its incidence rate.<sup>1</sup> The 2020 GLOBOCAN statistics on cancer demonstrated that around 19.3 million new cases of cancer and 10 million cancer-related deaths occurred in the year 2020.<sup>2</sup> The World Health Organization (WHO) reported that when assessing Disability-Adjusted Life Years (DALYs) specific to causes across all human diseases, cancer imposed the greatest social and economic burden on a global scale, accounting for 244.6 million DALYs in both men and women.<sup>1,3</sup> Multiple factors are reported to be responsible for the worldwide increase in cancer incidence rate and mortality, including lifestyle

modification (unhealthy diet, alcohol, smoking and sedentary lifestyle), exposure to carcinogens and pollutants, aging and growth of populations, exposure to infectious agents and socioeconomic development status.<sup>2,4</sup>

Although the spectrum of treatment for combating cancers has been broadened and improved in recent decades, surgery, followed by adjuvant radiotherapy, chemotherapy, immunotherapy, and targeted therapy are the most common treatment protocol for most cancers. These treatment protocols, however, have not always been totally effective due to poor response by patients, severe side effects of the therapies, and development of intrinsic and acquired resistance to the therapies, leading to relapse and recurrence. Recurrence due to drug resistance is widespread in cancers, with evidence indicating that the rate of recurrence varies in patients with non-small cell lung cancer (30–55%), soft tissue carcinoma (15–40%), ovarian cancer (85%), pancreatic cancer (37%) and glioblastoma (>55%).<sup>5–9</sup> It is well known that tumor cells are able to induce several survival mechanisms to drive resistance to various treatments. These mechanisms include alteration in drug target, increased efflux of therapeutic drug, DNA damage repair, altered metabolism, intra-tumor heterogeneity, and activation of signaling pathways that promote tumor cells survival and suppress tumor cell death among others.<sup>10–12</sup>

Post-operative adjuvant treatment for most cancers such as radiotherapy, chemotherapy and immunotherapy is based on the formation of reactive oxygen species (ROS) to induce tumor cells death. Therefore, the role of antioxidant response as a mechanism of resistance to post-operative adjuvant cancer therapies is now becoming clearer. The nuclear factor (erythroid-derived 2)-like 2 (Nrf2) is a Cap“n”Collar (CNC), basic leucine zipper (bZIP) transcription factor recognized as the primary controller of redox homeostasis in cells. In carcinogenesis, Nrf2 is reputed to possess a good and a bad side, thereby playing a dual role. Evidence abounds indicating that Nrf2 activation protects cells by preventing chemical- and radiation-induced carcinogenesis.<sup>13,14</sup> It is reported that Nrf2 inhibits carcinogenesis when its downstream targets are expressed. These downstream targets scavenge ROS, repair oxidative damage and ensure quick enzymatic modification and elimination of chemical carcinogens.<sup>13</sup> Recent evidence in the last decade has revealed the existence of a ‘dark side’ to Nrf2 activation in carcinogenesis, in contrast to its protective role in normal cells. It is well recognized now that Nrf2 is highly over-expressed in different cancer types, including lung, breast, colorectal, ovarian, pancreatic, liver and leukemia among others.<sup>15–17</sup> Cancers with aberrant activation of Nrf2 are characterized by tumor phenotypes including facilitated tumor progression, metastasis, chemoresistance and radio-resistance.<sup>18</sup>

Photodynamic therapy (PDT) is an innovative and minimally invasive therapeutic approach employed for either curative or palliative treatment in early-stage and late-stage cancers, respectively.<sup>19</sup> During PDT, light exposure activates a non-toxic photosensitizing agent administered to the patient, leading to the production of cytotoxic ROS in the presence of molecular oxygen. These reactive species are utilized to eliminate tumor cells. PDT presents several advantages over traditional cancer treatments, such as minimal systemic toxicity, selective tumor destruction capability, absence of severe side effects, and the ability for repeat procedures. Therefore, it can be applied either alone or in combination with other treatments, such as surgery, radiotherapy, chemotherapy, and immunotherapy. Clinically, PDT has received approval and demonstrated successful application in treating conditions such as non-melanoma skin cancers, cutaneous T cell lymphoma, biliary tract cancer, esophageal cancer, as well as squamous cell carcinoma of the head, neck, and bladder.<sup>20–23</sup> Despite the overwhelming effectiveness of PDT in most solid tumors, there are indications that some cancers are relatively recalcitrant, and some initially responsive cancers may develop acquired resistance to PDT. Resistance to PDT has been attributed to sub-optimal accumulation of photosensitizers (PS) in the tumor, insufficient ROS production due to poor photophysical and physicochemical properties of PS, and PDT-induced ROS generation resulting in the activation of survival signal transduction pathways.<sup>19,24</sup> Similar to what is obtained for other therapeutic modalities, activation of survival pathways has emerged as a major mechanism of resistance to PDT. It is now known that post-PDT, tumor cells activate signal transduction pathways that mediate PS extrusion, pro-survival autophagy, DNA damage repair, as well as up-regulated antioxidant response to mitigate PDT efficacy.<sup>25</sup> Although the mechanism underlying tumor resistance to PDT is not fully understood, we present in this review, the current knowledge regarding tumor resistance to PDT with particular focus on Nrf2 signaling pathway(s) and its role in mediating antioxidant response up-regulation and induction of drug efflux transporters in PDT treated tumors.

## Cancers and Oxidative Stress-Based Therapies

Reactive species (ROS) are natural byproducts of cellular metabolism, possessing the potential for both beneficial and harmful effects. ROS generated during normal physiological and cellular reactions are usually neutralized by enzymatic antioxidants, such as superoxide dismutase (SOD), glutathione peroxidase (GPx), and catalase (CAT), as well as non-enzymatic antioxidants including glutathione (GSH), thioredoxin (Trx), peroxiredoxin (Pdx) and glutaredoxin (Gdx). When present in low to moderate concentrations, ROS function as second messengers in cell signaling, overseeing various cellular processes such as proliferation, differentiation, cell cycle progression, growth arrest, apoptosis, modulation of enzyme activity, mediation of inflammation, and the elimination of pathogens and foreign particles. An imbalanced defense mechanism of antioxidants, an overabundance of reactive species, or the influx of free radicals from the environment can lead to an excessive accumulation of reactive oxygen species (ROS). This condition, known as oxidative stress, poses a harmful threat, potentially causing damage to vital cell structures such as lipid membranes, proteins, and nucleic acids. The prolonged presence of elevated oxidative stress has been widely acknowledged as a contributing factor to the pathophysiology of various diseases, including cancers.<sup>26</sup> Elevated levels of reactive oxygen species (ROS) and the resulting oxidative stress can give rise to unfavorable outcomes, including the activation of oncogenes, genomic instability, de-differentiation, and increased mitogenesis. These events have all been linked to the initiation, development, progression, invasion, and metastasis of cancer.<sup>26</sup>

While tumor cells are known to constitutively display a pro-oxidative shift in comparison with normal cells, the precise mechanism leading to this elevated oxidative stress in malignant cells and solid tumors remains unclear. However, malfunction of the mitochondrial respiratory chain, oncogenic activation and transformation, aberrant metabolism, inactivation of tumor suppressors, and a dysfunctional antioxidant response have been suggested as contributing to the elevated oxidative stress observable in malignant cells.<sup>27</sup> The heightened production of reactive oxygen species (ROS) serves as the origin of DNA-damaging agents in cancer cells, potentially fostering genetic instability. Simultaneously, ROS-induced mitochondrial dysfunction modifies the cellular apoptotic response to anticancer agents, contributing to the development of drug resistance in tumor cells.<sup>27,28</sup> Despite the deleterious effects of elevated ROS in cancer cells, recent advances have exploited this feature to develop innovative therapeutic strategies for selectively targeting and killing cancer cells. Non-surgical cancer treatment approaches, such as chemotherapy, radiation therapy and photodynamic therapy, shared a common mechanism of ROS production. The approach involves subjecting cancer cells to intensified ROS attack induced by these ROS-generating procedures. The aim is to overwhelm the cellular antioxidant defense, pushing cancer cells beyond the tolerable ROS threshold that distinguishes cell survival from cell death. Cancer cells, owing to their elevated intrinsic oxidative state, require less additional ROS to surpass the threshold leading to cell death compared to normal cells. This is attributed to the higher oxidative status of cancer cells. However, it is suggested that ROS levels that can kill tumor cells have a less drastic impact on normal cells because normal cells operate at lower oxidative status and possess efficient ROS-induced injuries repair mechanisms.<sup>26</sup> Because ROS induced toxicity to both cancerous and non-cancerous tissues, a major concern in the employment of oxidative stress-based therapy in cancers is how to target and kill tumor cells selectively and effectively. Thus, normal tissues will be better protected from harmful effect of therapeutic ROS if oxidative stress-based therapies are precisely targeted to the diseased tumor cells, as is the case with photodynamic therapy.

## Photodynamic Therapy as an Oxidative Stress Event

PDT is a minimally invasive treatment modality that has been approved for clinical use, with demonstrated effectiveness against various cancer types. The process entails the introduction of a photosensitizing drug via intravenous, systemic, or topical means, with a preference for localization within the targeted tumor. Subsequent exposure of the tumor-localized photosensitizer to visible light, at a wavelength corresponding to the absorption peak of the PS, triggers the production of cytotoxic reactive oxygen species in the presence of molecular oxygen. These ROS generated act to eliminate tumors through direct cell killing, damage to tumor-associated vasculature, or the activation of an anti-tumor immune response.<sup>29-31</sup> PDT can induce direct killing of tumor cells through the three primary mechanisms of apoptosis, necrosis, and autophagy-associated cell death. In vitro studies have identified apoptosis as the primary mode of cell death after

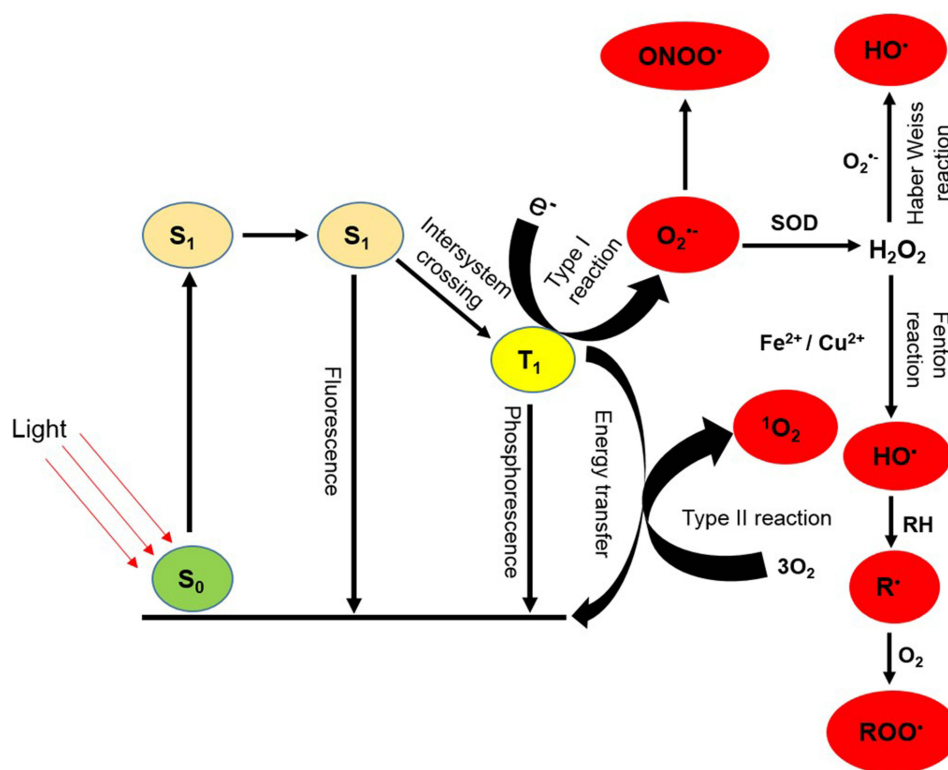
PDT. However, evidence has shown that factors such as the subcellular localization of the photosensitizer (PS) in various organelles (eg, lysosome, mitochondria, melanosome, and endoplasmic reticulum), the dose of PDT (PS concentration  $\times$  light fluence), drug-light interval (DLI), glucose deprivation, and cell genotype all play pivotal roles in determining the predominant mechanism of cell death.<sup>32–36</sup> The effectiveness of PDT is dependent on the simultaneous presence of three essential components: a photosensitizer, molecular oxygen, and a visible light source. Photosensitizers play a crucial role in the efficacy of PDT. For a PS to be ideal for photodynamic therapy, it must be chemically pure and stable, accumulate preferentially in and be retained by the target tumor, must not promote dark cytotoxicity, be rapidly cleared from normal tissue, and must possess considerable triplet quantum yield, contributing to the production of ROS after light exposure.<sup>31,32</sup>

## Photochemical Mechanisms of ROS Production in PDT

The photosensitizer (PS) molecule initially exists in a singlet ground state ( $S_0$ ), with paired electrons exhibiting opposite spins in a low-energy molecular orbital. When irradiated with quantum light, the PS absorbs photon leading to the formation of an excited singlet state ( $S_1$ ). The  $S_1$  state, characterized by the excitation of one electron into a higher-energy molecular orbital, is inherently unstable and short-lived, with several potential fates. The  $S_1$  state PS may decay back to  $S_0$  undergoing molecular relaxation and internal conversion resulting in the emission of a photon (fluorescence) and the generation of heat, respectively. In most PS, the energy of the photon is redistributed over two unpaired electrons with the same spin orientation, causing the  $S_1$  to undergo intersystem crossing to a longer-lived triplet state ( $T_1$ ) characterized by lower energy. In the  $T_1$  state, the PS takes advantage of its longer lifetime to interact with surrounding molecules, and it is well established that generation of cytotoxic species produced during PDT occurs in this state. The PS in the  $T_1$  state can undergo two types of reactions. It can undergo a direct reaction with biomolecules in its vicinity, transferring a proton to form a radical anion or generate a radical cation as a result of the transfer of electron in what is referred to as a type I reaction. The radicals generated can interact with molecular oxygen to form superoxide anion ( $O_2^{\cdot-}$ ), the primary ROS produced in type I reaction. Type II reaction occurs when there is a direct transfer of energy from the  $T_1$  photosensitizer to molecular oxygen in its natural triplet state ( $^3O_2$ ) to generate highly reactive singlet oxygen ( $^1O_2$ ) (see Figure 1). While the type II reaction, which generates singlet oxygen, is considered the major cytotoxic pathway in PDT, evidence suggests that both types of reactions can occur at the same time during PDT. The ratio of these processes depends on factors such as the type of photosensitizer used, substrate concentration, and oxygen levels.<sup>30,37</sup> Furthermore, reports from other studies also revealed that the high efficacy of PDT results from synergistic action of ROS ( $O_2^{\cdot-}$ ,  $HO^{\cdot}$  and  $^1O_2$ ) generated during type I and II reactions.<sup>29,38</sup>

Superoxide ( $O_2^{\cdot-}$ ) radical produced in type I reaction is a relatively stable and not particularly reactive in biological systems. It does not by itself cause direct oxidative damage to nucleic acids, lipids or carbohydrates. It can undergo a dismutation reaction with itself, catalyzed by the enzyme superoxide dismutase (SOD) to generate  $H_2O_2$ , a non-radical molecule required for the functioning of many enzymes. Though not in itself highly cytotoxic,  $O_2^{\cdot-}$  is able to generate highly cytotoxic secondary products by reacting with other biologically relevant radicals.  $O_2^{\cdot-}$  can interact with  $H_2O_2$  through a Haber-Weiss or an iron- or copper-mediated breakup of  $H_2O_2$  (Fenton reaction), giving rise to the formation of the highly reactive and cytotoxic  $HO^{\cdot}$  radical.<sup>39</sup> Furthermore,  $O_2^{\cdot-}$  can react with  $HO^{\cdot}$  to form  $^1O_2$  or with nitric oxide ( $NO^{\cdot}$ ) to generate peroxynitrite ( $ONONO^{\cdot}$ ) another cytotoxic product (Figure 1).  $HO^{\cdot}$  damage is diffusion rate-limited and due to its extremely high reactivity, will damage biomolecules in close proximity to its site of formation. It can add itself to and/or abstract an electron from an organic substrate such as fatty acid side chain of membranes forming a hydroxylated adduct or an oxidized substrate, respectively.<sup>37</sup> Both products are also radicals and initiate a chain reaction when they react with other molecules such as oxygen in the ground state to generate a peroxy radical ( $ROO^{\cdot}$ ) which further interacts with another organic substrate propagating a chain reaction.

$^1O_2$  and  $HO^{\cdot}$  are the two most important ROS generated during PDT because of their extreme reactivity towards biomolecules. Although both and other ROS are short-lived ( $^1O_2 < 40$  ns and  $HO^{\cdot} < 1$  ns), evidence has shown that PDT induced prolonged oxidative stress in vitro.<sup>30,40–42</sup> One of the outcomes of ROS-mediated damage in PDT-treated cells is the peroxidation of membranous lipids. Despite the fact that lipids are less abundant than proteins, many factors make membranous organelles and cell membrane prime target for PDT-derived ROS. The cell membrane is particularly



**Figure 1** Photochemical generation of ROS during PDT. Upon absorption of light of appropriate wavelength by the PS, an electron is elevated to an excited singlet state ( $S_1$ ) from the singlet ground state ( $S_0$ ). This excited singlet state is transient and can release energy through photon emission (fluorescence). Alternatively, it may undergo intersystem crossing, leading to the formation of a long-lived excited triplet state ( $T_1$ ). The  $T_1$  state is responsible for generating reactive species, including  $O_2^{\cdot-}$ ,  $H_2O_2$ ,  $HO^{\cdot}$  and  $^1O_2$  through both type I and type II reactions.

**Abbreviations:**  $H_2O_2$ , hydrogen peroxide;  $HO^{\cdot}$ , hydroxyl radical;  $ONOO^{\cdot}$ , peroxynitrite radical;  $^1O_2$ , singlet oxygen;  $O_2^{\cdot-}$ , superoxide radical; R, alkyl radical;  $ROO^{\cdot}$ , peroxy radical;  $S_0$ , singlet ground state;  $S_1$ , excited singlet state;  $T_1$ , excited triplet state.

vulnerable to damage from reactive oxygen species (ROS) due to the high susceptibility of polyunsaturated fatty acid (PUFA) residues in phospholipids to oxidation. In addition, molecular oxygen ( $O_2$ ) is highly soluble in the lipid environment, this increases the likelihood of an excited photosensitizer (PS) encountering  $O_2$  and generating singlet oxygen ( $^1O_2$ ) and other ROS, especially compared to a hydrophilic environment.<sup>29</sup> ROS produced during photodynamic therapy (PDT) can initiate lipid peroxidation by abstracting hydrogen from lipids or, in the case of  $^1O_2$ , by directly adding to mono- or polyunsaturated fatty acids, generating a lipid radical that stabilizes itself by reacting with oxygen to form a peroxy radical. This peroxy radical then attacks a neighboring fatty acid, leading to the creation of a stable lipid hydroperoxide and simultaneously generating another lipid radical. This chain reaction propagates, ultimately causing the destruction of lipid bilayers in membranes.<sup>39,43</sup> Lipid hydroperoxides can undergo conversion into unstable conjugated dienes, which in turn decompose into various chemically reactive and biologically active products, including malondialdehyde (MDA), trans-4-hydroxy-2-nonenal, and 2-propanal (acrolein). These products resulting from lipid peroxidation have the capacity to modify macromolecules, disrupt metabolism, and influence cell signaling pathways, ultimately culminating in tumor cell destruction.

Proteins, being central to the majority of cellular functions, are abundant in biological systems, and consequently represent crucial targets for reactive oxygen species (ROS) generated through photodynamic therapy (PDT).  $HO^{\cdot}$  initiates the attack on the polypeptide backbone via an abstraction of the  $\alpha$ -hydrogen atom from an amino acid residue leading to the formation of a carbon-centered radical. In the presence of  $O_2$ , this radical evolves into a peroxy radical, which, upon interaction with the protonated form of superoxide, is further transformed into alkyl peroxides.<sup>39,44</sup> While PDT-derived ROS can target all amino acids in protein, cysteine, because of its thiol group exhibits the highest susceptibility. Nonetheless, though generally to a lesser degree than cysteine, methionine, tyrosine, and tryptophan are also prone to photo-oxidation.<sup>45</sup> ROS attack on proteins can lead to the oxidation of amino acid residue side chains, as well as the

oxidation of the protein backbone, resulting in the formation of protein–protein cross-linkages. Although PDT-derived ROS attack on proteins primarily resulted in detrimental effects on protein function, disruption of normal redox homeostasis in photosensitized cells also induced cell death.

Reactive oxygen species-induced damage to DNA is an important cause of cell death during PDT. The HO<sup>•</sup> radical, produced via type I reaction during PDT is the most destructive reactive species. It has the capability to interact with and modify all components of the DNA molecule, including the purine and pyrimidine bases, as well as the deoxyribose sugar backbone. This interaction results in various forms of damage such as base or sugar lesions, single-strand breaks, double-strand breaks, abasic site formation, and the formation of DNA–DNA or DNA–protein cross-links. DNA damage has been reported in many cancer cells post-PDT. The formation of the double-strand breaks (DSBs) marker,  $\gamma$ -H2AX has been reported in HaCAT keratinocytes and WM35 melanoma cells.<sup>46,47</sup> Singlet oxygen exhibits selectivity in its oxidative modification of DNA, with guanine being the most susceptible organic base to <sup>1</sup>O<sub>2</sub> oxidation. This susceptibility leads to the formation of 8-oxo-7,8-dehydro-2-deoxyguanosine,<sup>48</sup> a product that has been identified following photodynamic therapy.<sup>49</sup> Additionally, aldehydic products of lipid peroxidation, such as acrolein and 4-HNE, can also attack and modify DNA. This process results in the creation of bulky exocyclic adducts, which can contribute to DNA–DNA and DNA–protein crosslinking, ultimately impairing the binding of transcription factors. Although cells are equipped with the capability to repair oxidative DNA damage, however, unrepaired damage prior to DNA replication may induce cell death, DNA mutation, replication errors and genomic instability in photosensitized tumors leading to eventual tumor destruction.

## ROS-Mediated Cytotoxicity to Tumor Cells After PDT

Singlet oxygen and other cytotoxic photoproducts generated upon irradiation during PDT start a cascade of biochemical events that may induce damage and kill tumor cells via three main biological mechanisms. These include (1) direct killing of tumor cells (2) destruction of tumor-associated vasculature and (3) stimulation of anti-tumor immune response. While all three processes have been implicated in the overall outcome of PDT effect, the relative contribution of each process is difficult to pinpoint. Therefore, the focus of this review will be on PDT-induced direct killing of tumor cells.

Direct killing of tumor cells in PDT is mediated via three different processes of apoptosis, necrosis and autophagy. This differential direct cell killing is known to depend on many factors including physicochemical characteristic and subcellular localization of PS, overall PDT dose (PS concentration vs light fluence), as well as drug-light interval (DLI). Many reports have shown that photosensitizers localize in different organelles (mitochondrion, lysosome, ER, melanosome and plasma membrane), and this is crucial in determining the predominant mechanism of cell death.<sup>32,35,50</sup> The subcellular localization of a PS may be determined by its ionic status, with cationic photosensitizers accumulating in the mitochondrion, while anionic ones accumulate in the lysosome.<sup>37,50</sup> All other conditions being equal, it is generally accepted that photo-oxidative reaction with photosensitizers localizing to the mitochondria lead to apoptosis, while PDT with compounds targeting the cytoplasmic membrane, lysosomes, ER or Golgi membrane leads to necrosis. The cell death mechanism activated following PDT may also be a function of the quantity and type of ROS generated, as well as the degree of oxidative damage. Many reports have established that mild oxidative damage increases the likelihood of cells dying by undergoing apoptosis. In contrast, severe damage hinders ATP production and the activation of apoptotic pathways, compelling the cell to undergo necrosis.<sup>29,51</sup> Whether PDT elicits an apoptotic, necrotic or autophagic cell death may also be cell type-specific. For example, Davids et al<sup>52</sup> demonstrated that hypericin-mediated PDT triggers distinct mechanisms of cell death in melanoma cells that are dependent on the presence or absence of melanin. The study revealed that in melanocytes and pigmented UCT-MEL 1 cells, a necrotic mode of cell death was induced by hypericin-mediated PDT, while apoptosis is the cell death mechanism triggered in keratinocytes and unpigmented A375 cells. In a study with two variants of mouse B16 melanoma cell line, Sparsa et al<sup>53</sup> reported that 5-ALA-mediated PDT induced a p53-dependent apoptotic cell death in pigmented B16F10 cells and an autophagic response leading to a caspase-independent cell death in unpigmented B16G4F cells. Apoptotic cell death mediated by PDT may be via the extrinsic (activation of death receptors) or intrinsic (mitochondrial outer membrane permeabilization and subsequent release of cytochrome C) pathway. Whichever pathway dominates may also be a reflection of many factors, including type of PS and cell type. The effectiveness of photo-activated hypericin in killing different melanoma cell types was reported by

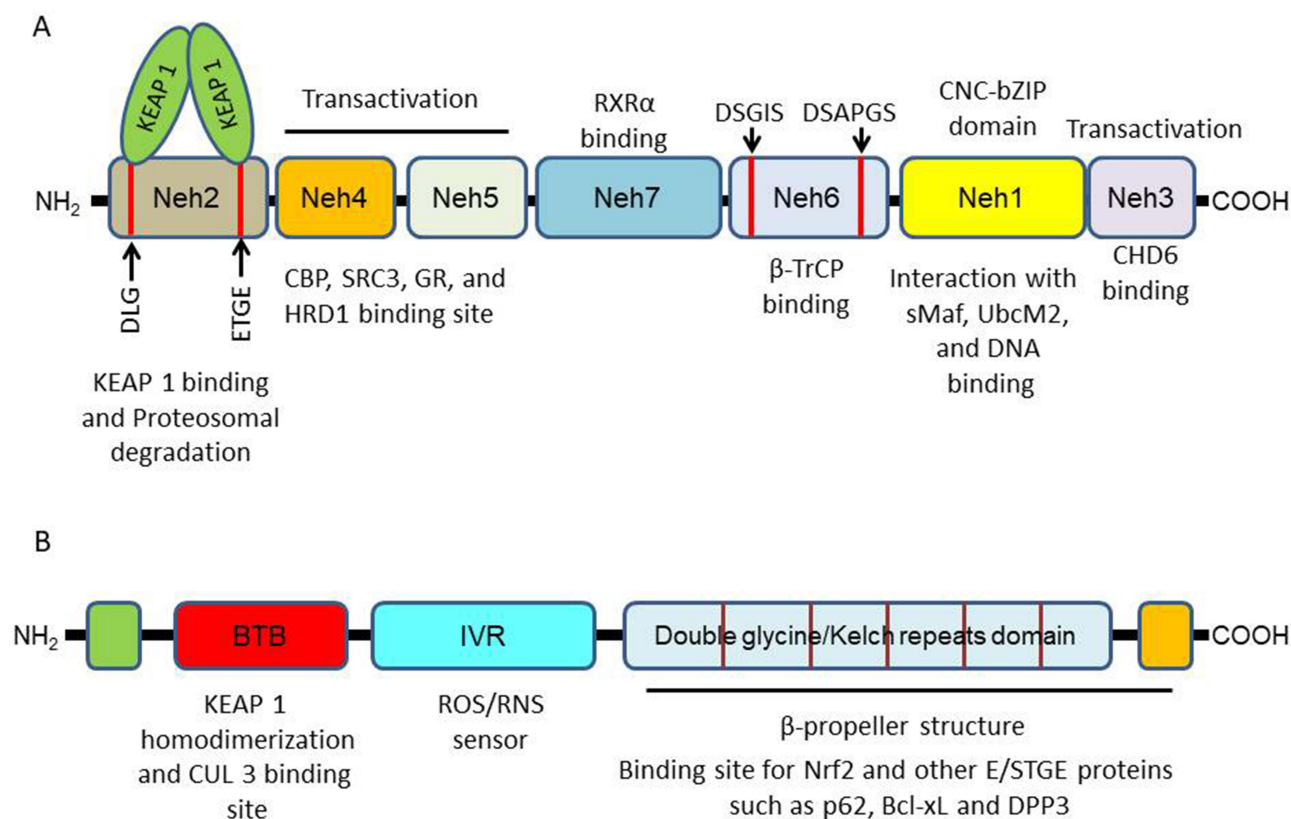
Kleemann et al.<sup>35</sup> In the study, photo-activation of hypericin resulted in an extrinsic (A375, unpigmented) and intrinsic (UCT-MEL 1, pigmented) caspase-dependent apoptotic mode of cell death, as well as a caspase-independent apoptotic mode not involving apoptotic-inducing factor in 501 Mel melanoma cells. While the exact mechanistic pathways responsible for the elimination of apoptotic tumor cells following PDT are not fully understood, there is a broad agreement that the irradiation of a PS triggers intrinsic (mitochondrial-dependent) apoptosis through the classical pathway. This process involves inducing photo-damage and deactivating the anti-apoptotic Bcl-2 protein family, consequently causing the pro-apoptotic Bax and Bak proteins to translocate into the outer mitochondrial membrane. The resulting permeabilization of the mitochondrial membrane stimulates the release of cytochrome c, initiating a cascade-like activation of effector caspases. These activated caspases then propel the cell through an irreversible process leading to apoptosis.

Many studies have shown that autophagy is triggered in tumor cells during PDT, however it is still unclear if the process is a pro-death or a cytoprotective mechanism. Buytaert et al.<sup>54</sup> reported that PDT can stimulate an autophagic cell death mechanism, at least under conditions of apoptosis inhibition in Bax<sup>-/-</sup> and Bak<sup>-/-</sup> murine embryonal fibroblasts (MEFs) subjected to hypericin-mediated PDT. Davids et al.<sup>55</sup> additionally documented the activation of a mechanism of cytoprotective autophagy in melanoma cells as a response to oxidative stress induced by hypericin-mediated PDT. Cancer cells may respond to oxidative stress-based therapy including PDT, chemotherapy, and radiotherapy by initially inducing autophagy. However, persistent oxidative stress can overwhelm the cells' endogenous antioxidant defense capacity, resulting in a transition from autophagy to a potential senescent phenotype in an effort to prolong cellular survival. This transition may ultimately lead the cells to die either by apoptosis or necrosis.<sup>56-58</sup> Additional evidence revealed that autophagy may contribute to PDT-induced apoptosis, with the two processes occurring independently of one another,<sup>59</sup> or simultaneously.<sup>60,61</sup> Overall, the emerging consensus is that apoptotic, necrotic, and autophagic cell death may be induced by PDT in tumor cells and that the predominating pathway is a function of many variables including cell type, the type of PS, its physico-chemical properties and intracellular localization, light dose (local PS concentration vs fluence), drug-light interval (DLI), as well as oxygen concentration in tissues.

## KEAP1/Nrf2 Signaling Pathway

Nrf2 (encoded by the gene, NFE2L2), a basic region-leucine zipper (bZIP)-type factor belonging to the Cap 'n' Collar (CNC) transcription factor family, plays an important role in the regulation of redox, protein and metabolic homeostasis.<sup>62</sup> Having a molecular weight of 68 kDa, Nrf2 is a protein containing 605 amino acids. Structurally, Nrf2 is composed of seven (7) highly conserved Nrf2 erythroid-derived cap "n" collar homology (ECH; Neh) domains (Neh1-7; **Figure 2A**). Neh1 is a CNC-bZIP domain, and it plays several important roles. It is the region that is necessary for DNA binding at antioxidant response element/electrophile response element (ARE/EpRE) of gene promoters, as well as interaction with small MAF proteins.<sup>63,64</sup> The domain is also important as it regulates the stability of Nrf2 by its association with UbcM2, a ubiquitin-conjugating enzyme.<sup>64,65</sup> Neh2 domain is located at the N-terminal, and it contains DLG and ETGE amino acid motifs that are critical for binding to KEAP1 (Kelch-like ECH-associated protein 1), and seven ubiquitin-lysine rich residues for targeting Nrf2 for ubiquitination and proteosomal degradation.<sup>64,66</sup> Neh3 domain found at the C-terminal, and Neh4 and Neh5 domains are all independent transactivation domains. A region within Neh3 has been reported to be associated directly with chromodomain helicase DNA-binding protein 6 (CHD6).<sup>67</sup> Both Neh4 and Neh5 domains of Nrf2 contain acid-rich residues where the cooperative binding of co-activators (such as CREB-binding protein (CBP) and steroid receptor co-activator 3 (SRC3) and/or co-repressors (such as glucocorticoid receptor; GR, and 3-hydroxy-3-methylglutaryl reductase degradation; HRD1)) take place to mediate activation and repression of Nrf2 signaling, respectively.<sup>64,68-72</sup> Neh6 is a serine rich domain, and it is composed of DSGIS and DSAPGS amino acid motifs where  $\beta$ -transducin repeat-containing protein ( $\beta$ -TrCP) binds to promote Nrf2 ubiquitination and proteosomal degradation.<sup>64,73,74</sup> Direct binding of retinoid X receptor-alpha (RXR $\alpha$ ) takes place at the Neh7 domain leading to repression of the transcriptional activation of Nrf2.<sup>75</sup>

KEAP1, a dimeric protein primarily located in the cytoplasm, stands out as the most extensively studied intracellular regulator of Nrf2. Structurally, it consists of five conserved domains (**Figure 2B**) namely, an N-terminal region (NTR), a conserved Broad-Complex/TramTrack/Bric-a-Brac (BTB) domain, an intervening region (IVR), a double glycine repeat (DGR) domain (comprising six subdomains, also known as the Kelch repeats domain), and a C-terminal region



**Figure 2** Protein structure of (A) Nrf2 and (B) KEAP1 showing different domains.

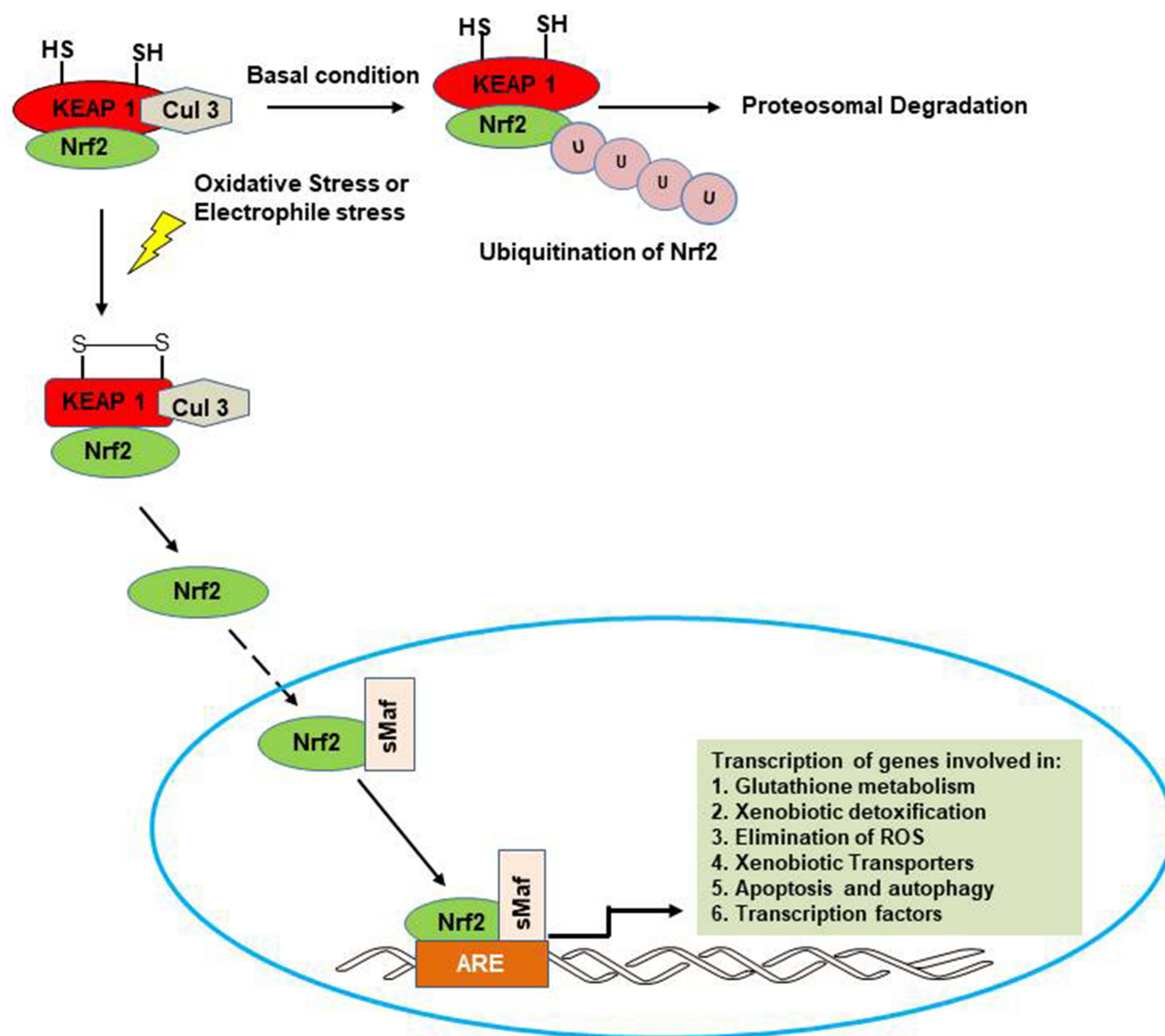
**Abbreviations:** BTB, broad-Complex/TramTrack/Bric-a-Brac domain; bZIP, basic region-leucine zipper; CBP, CREB-binding protein; CHD6, chromodomain helicase DNA-binding protein 6; CNC, cap 'n' collar; CUL3, Cullin 3; DPP3, dipeptidyl peptidase 3; GR, glucocorticoid receptor; HRD1, 3-hydroxy-3-methylglutaryl reductase 1; IVR, intervening region; KEAP1, Kelch-like ECH-associated protein 1; Neh, Nrf2-ECH homology domain; Nrf2, Nuclear factor (erythroid-derived 2)-like 2; RNS, reactive nitrogen species; ROS, reactive oxygen species; RXR $\alpha$ , retinoid X receptor-alpha; sMaf, small musculo-aponeurotic fibrosarcoma protein; SRC3, steroid receptor co-activator 3; UbcM2, ubiquitin conjugating enzyme;  $\beta$ -TrCP,  $\beta$ -transducin repeat-containing protein.

(CTR).<sup>64,76</sup> The BTB domain is crucial for KEAP1 homodimerization and interaction with Cullin E3 ubiquitin ligase (CUL 3), a pivotal event for Nrf2 ubiquitination.<sup>77,78</sup> The IVR domain, rich in cysteine, operates as a redox stress sensor.<sup>79,80</sup> The DGR/Kelch repeats domain, in conjunction with the CTR, forms a six-bladed propeller structure necessary for binding with the Neh2 domain of Nrf2 and other proteins with ETGE and/or STGE conserved motifs, including p62, Bcl-xL, and dipeptidyl peptidase 3.<sup>64,81–83</sup>

## Physiological Role of Nrf2

Nrf2 has historically functioned as the primary controller of the expression of cytoprotective genes, specifically those involved in antioxidant and detoxification processes. Nonetheless, emerging evidence suggests that Nrf2 possesses additional functions beyond its role in regulating redox homeostasis. In normoxic environments, Nrf2 typically remains confined to the cytoplasm, where it forms a complex with its inhibitory partner, KEAP1. KEAP1 functions as a substrate adaptor for the Cullin 3-based E3 ubiquitin ligase enzyme complex (Cul 3), facilitating the ubiquitination of Nrf2. This ubiquitination leads to the subsequent proteasomal degradation of Nrf2, thereby maintaining its basal levels at a minimum, and preventing the induction of target genes downstream of Nrf2. In the presence of a stressor such as ROS and small-molecule electrophiles, oxidation of the sulfhydryl groups in the Nrf2-binding domain of KEAP1 takes place. This led to a change in conformation which impairs the binding of Nrf2 to KEAP1. Consequently, the ubiquitination of Nrf2, and its subsequent proteasomal degradation is stopped. Thus, Nrf2 accumulates in the cytoplasm from where it is eventually translocated into the nucleus (Figure 3). Once in the nucleus, Nrf2 heterodimerizes with the small musculo-aponeurotic fibrosarcoma (Smaf) protein. Subsequent binding of the Nrf2-sMAF complex to the antioxidant or electrophile response element (ARE/EpRE) sequence orchestrated the





**Figure 3** Activation and response of Nrf2. Under normal condition, KEAP1 binds Nrf2 and package it for ubiquitination and proteosomal degradation with the help of Cullin 3-E3 ubiquitin ligase. In the presence of a stressor such as ROS and electrophiles, Nrf2 is activated and translocated into the nucleus where its binding to the antioxidant response element sequence leads to transcription of different cytoprotective genes.

**Abbreviations:** ARE, antioxidant response element; CUL 3, Cullin 3-E3 ubiquitin ligase; Keap1, Kelch-like ECH-associated protein 1; Nrf2, Nuclear factor (erythroid-derived 2)-like 2; sMaf, small musculo-aponeurotic fibrosarcoma protein.

transcriptional regulation of numerous downstream target genes crucial for preserving cellular redox homeostasis.<sup>84,85</sup> The downstream targets activated by Nrf2 include genes involved in (i) glutathione biosynthesis (glutamate cysteine ligase modifier subunit, GCLM; glutamate cysteine ligase catalytic subunit, GCLC; glutathione peroxidases, GPxs; glutathione reductase, GR), (ii) xenobiotic metabolism (glutathione-s-transferases, GSTs; microsomal epoxide hydroxylases, EH-1; NAD(P)H: quinone oxidoreductase 1, NQO1; and heme oxygenase-1, HO-1), (iii) scavenging of ROS (superoxide dismutase, SOD; catalase, CAT; thioredoxin reductase 1, Trxn 1; sulfiredoxin 1, Srxn 1; and peroxiredoxin 1, Prdx 1), (iv) drug efflux (multi-drug resistance protein (Mdrp) gene family), and (v) apoptosis and autophagy (p62, Bcl-2 and Bcl-xL).<sup>86</sup>

While the accepted cellular trigger for Nrf2 activation involves modifying the cysteine residues of KEAP1 through chemical adduction, oxidation, nitrosylation, or glutathionylation, emerging evidence suggests that Nrf2 activation can be regulated through alternative mechanisms. These include the phosphorylation of Nrf2 by upstream protein kinases such as PKC, PI3K/Akt, GSK-3 $\beta$ , and JNK, the binding of other proteins like caveolin and p21 to Nrf2, epigenetic modification of Nrf2 by microRNAs such as miR-144, miR-28, and miR-200a, as well as the direct interaction of

heme with Bach1, facilitating the heterodimerization of Nrf2 with sMAF. These diverse mechanisms have all been reported to contribute to the repression and/or activation of Nrf2.<sup>87,88</sup>

## Tumor Suppression Role of Nrf2 in Cancer

Because of the cytoprotective functions of target genes downstream of Nrf2, it is usually regarded as a vital controller of cellular defense against ROS- and electrophile-induced stress. The target genes expressed as a result of Nrf2 activation code for various enzymes that are involved in processes such as scavenging ROS and RNS, xenobiotic detoxification, xenobiotic efflux, apoptosis and autophagy among others. Normal functioning of the Nrf2/KEAP1 signaling pathway is vital to inhibiting oxidative stress and prevents damage to tissues and organs. Research findings have shown that mutations resulting in diminished Nrf2 activity can foster oxidative and inflammatory damage, consequently contributing to the promotion of various conditions like cardiovascular disorder, cancer, diabetes, neurodegenerative disease, and aging.<sup>89</sup> Reports showing that induction of Nrf2 can suppress tumorigenesis, especially in its early stages, are available in literature. Many studies using chemical agents, as well as genetic manipulations that promote Nrf2 activity, have demonstrated the suppression of the tumorigenesis process.<sup>90</sup> The anti-cancer properties of synthetic and naturally-occurring chemopreventive substances, which are known inducers of Nrf2, are well reported. Sulforaphane, a natural product obtained from broccoli, has been shown to inhibit the promotion and progression stage of tumorigenesis in different types of cancers via the activation of Nrf2/ARE-mediated genes.<sup>14,91–93</sup> Using a *LSL-Kras<sup>G12D/+</sup>;LSL-Trp53<sup>R172H/+</sup>;Pdx-1-Cre triple mutant* (KPC) transgenic mouse model of pancreatic cancer, Liby et al,<sup>94</sup> reported that synthetic oleanane triterpenoids (potent chemopreventive and Nrf2 activity inducer) delay tumorigenesis and prolong survival by inhibiting the transcription of oncogenes including K-Ras and TP53 in the pancreas. Similarly, in a BRCA1 mutated mouse model of breast cancer, the same synthetic chemopreventive agent was reported to delay tumor development in the breast and extend survival BRCA1 mutated mice.<sup>95</sup>

Further evidence of the tumor suppression role of Nrf2 was obtained from studies involving the use of Nrf2 knockout mouse models. Carcinogens-treated Nrf2 deficient mice were reported to show increase susceptibility to carcinogenesis and develop large number of tumors in the liver, fore-stomach and urinary bladder compared with wild-type mice.<sup>96–99</sup> Furthermore, Nrf2 knockout mice were reported to show increased susceptibility to colitis-associated colorectal cancer in contrast to their genetically unaltered counterparts.<sup>100</sup> Similarly, evidence from studies employing Nrf2 knockout mouse model revealed that Nrf2 is essential for the chemoprevention efficacy of oltipraz, 1-[2-cyano-3,12-dioxooleana-1,9(11)-dien-28-oyl] imidazole, and sulforaphane in different carcinogen-induced carcinogenesis models.<sup>101–104</sup> Another piece of evidence by Satoh et al<sup>105</sup> indicated that Nrf2 deficiency produces a conducive microenvironment for cancer cells metastasis to the lung, while its overexpression as a result of KEAP1 knockout increased resistance to metastasis of cancer cells to the lung. A reduced expression of Nrf2 has been reported in individuals with single nucleotide polymorphism (SNP) in the Nrf2 promoter region, making such individuals susceptible to increased risk of lung cancer.<sup>106,107</sup> All these studies showed that Nrf2 may play an important role as an inhibitor of the tumorigenesis process and form the basis of the use of Nrf2 activators in clinical trials for the prevention of cancer and other diseases.

## Oncogenic Role of Nrf2 in Cancer

Many studies, including those discussed above, have shown that Nrf2 is essential for chemopreventive agents to block carcinogenesis and protect normal cells from stressors such as endogenously generated ROS, radiation, environmental toxin and xenobiotics.<sup>85,101,102</sup> Recent evidence, however, has indicated that the role of Nrf2 is more complicated with its reported role in promoting cancer cell survival and resistance in many types of cancer therapy. Many reports have suggested that Nrf2 and its downstream targets are constitutively up-regulated in many tumors and this confers a survival advantage to mammalian cells under adverse conditions.<sup>108–111</sup> Similarly, overexpression of Nrf2 is associated with poor prognosis in some tumors.<sup>112–114</sup> This constitutive overexpression has been adduced primarily to somatic gain of function mutations in Nrf2 or loss of function mutations in KEAP1 which has been reported in many human cancers, including lung adenocarcinoma,<sup>115,116</sup> hepatocellular carcinoma,<sup>117,118</sup> breast,<sup>119</sup> colorectal,<sup>120</sup> gall bladder,<sup>118</sup> ovarian carcinomas,<sup>112</sup> skin squamous cell carcinoma,<sup>121</sup> endometrial,<sup>122</sup> and papillary adenocarcinoma.<sup>123</sup> Other mechanisms that lead to the constitutive activation of the Nrf2-KEAP1 pathway have also been reported. They include epigenetic modifications in the KEAP1 promoter, negative

regulation of Nrf2-KEAP1 pathway by micro RNAs, aberrant accumulation of proteins disrupting Nrf2-KEAP1 interactions, as well as metabolism-induced KEAP1 modification.<sup>63</sup>

Nrf2 activation might promote tumorigenesis in different ways, including mediating tumor cell proliferation. It is reported that constitutive activation of Nrf2 in tumor cells leads to increased expression of metabolic enzymes such as glucose-6-phosphate dehydrogenase, phosphogluconate dehydrogenase and transketolase. These enzymes promote glucose and glutamine metabolism in the pentose phosphate pathway and contribute to purine and amino acid synthesis, all key events driving metabolic reprogramming for tumor cell proliferation.<sup>14,124</sup> There is evidence of a correlation between Nrf2 and tumor cell proliferation, with studies indicating that KEAP1<sup>-/-</sup> cell lines proliferate faster than their wild-type counterpart, while Nrf2<sup>-/-</sup> cells showed slower proliferation.<sup>13,109,125–127</sup> Furthermore, reduction in tumor cell proliferation associated with down-regulated Ki67 expression and p53-induced senescence has been reported with Nrf2 knockdown.<sup>13,128,129</sup> A study by Rojo et al<sup>130</sup> reported that loss of PTEN and subsequent inhibition of GSK-3, results in upregulation of PI3K-AKT and Nrf2 signaling, leading to increased proliferation rates and tumorigenicity in human endometrioid tumors. Functional crosstalk between oncogenic proteins (such as BRAF, KRAS and MYC) and Nrf2 signaling is another mechanism by which Nrf2 can promote cancer cell proliferation. Mutation in these oncogenic proteins is known to increase constitutive activation of Nrf2 in different tumors, including human pancreatic cancer.<sup>18</sup> Silencing Nrf2 in KRAS mutant cells inhibits oncogene-mediated proliferation and tumorigenesis in primary cells and tissues of mouse expressing KRAS<sup>G12D</sup> and BRAF<sup>V619E</sup> and in human pancreatic cancer, indicating that Nrf2 plays proliferative role in tumorigenesis.<sup>128</sup>

An important hallmark of cancers is their ability to evade apoptosis. Constitutive activation of Nrf2 promotes tumor development and progression by inhibiting apoptosis of cancer cells. Overexpression of Nrf2 in cancer cells results in transcription of gene coding for various cytoprotective enzymes which scavenge ROS and promote resistance to ROS-driven apoptosis. Interaction between Nrf2 and p53 signaling may also contribute to the ability of cancer cells to evade apoptosis. Over-activation of Nrf2 leads to transcription of downstream targets including x-CT, NQO1 and GST, all of which counteract the activity of p53, a tumor suppressor known for induction of cell growth arrest and apoptosis.<sup>14,131</sup> Similarly, Nrf2 downstream targets, glutathione-s-transferase pi 1 (GSTP1) and p62 prevent apoptosis by blocking the activation of c-Jun N-terminal kinase (JNK), a pro-apoptosis protein,<sup>132</sup> and initiating selective autophagy of KEAP1.<sup>14,133</sup> Further evidence from a study in mouse hepatoma (Hep1) and human hepatoblastoma (HepG2) cells revealed that Nrf2 induced an overexpression of Bcl-2 (B-cell lymphoma 2), leading to a repression in etoposide-induced apoptosis and increased cell survival.<sup>134</sup>

Another mechanism through which Nrf2 contributes to tumorigenesis is via its role in metastasis. Constitutive Nrf2 activation in pancreatic adenocarcinoma cells is reported to promote epithelial-to-mesenchymal (EMT) transition and invasion by lowering the expression of E-cadherin.<sup>135</sup> Silencing of Nrf2 suppresses radiation-induced invasion and migration by up-regulating E-cadherin expression and down-regulating N-cadherin expression in non-small cell lung cancer.<sup>136</sup> Further evidence revealed that constitutively activated Nrf2 suppressed the degradation of Bach 1, a pro-metastatic transcription factor, thereby promoting lung cancer metastasis.<sup>18,137</sup> In addition, Nrf2 over-activation promotes proliferation and metastasis in MCF-7 and MDA-MB-231 human breast cancer cell lines by increasing RhoA gene expression.<sup>127</sup> It has also been reported that Nrf2 overexpression-induced metastasis is mediated via matrix metalloproteases, as evidence showed that deletion of Nrf2 mediated by shRNA repressed matrix metalloprotease 2 (MMP 2) expression and increased E-cadherin expression, leading to decreased invasion and migration in esophageal squamous cell carcinoma.<sup>138</sup> Surprisingly, a number of studies have shown that Nrf2 might as well play anti-metastatic role in cancers. During metastatic process in human prostate cancer, a reduction in the expression of Nrf2 and its target genes were reported.<sup>139</sup> Similarly, loss of Nrf2 increases migration, invasion and metastasis mediated by up-regulated SMAD signaling in hepatocellular carcinoma (HCC) cell lines.<sup>140</sup> In A549 lung cancer cell lines, Nrf2 inhibition facilitated TGF- $\beta$ 1-induced cell motility or migration via NOX4-ROS signaling.<sup>141</sup> These studies indicate that the pro- or anti-metastatic role of Nrf2 in cancer is not unequivocal, thus further studies are still warranted.

Development of intrinsic and acquired resistance to conventional treatments such as chemotherapy and radiation therapy is the most significant role of Nrf2 in tumorigenesis. Treatment of cancer cells with chemotherapeutic drugs including cisplatin, doxorubicin, etoposide, paclitaxel and others, as well as radiation therapy, induced higher ROS level to kill cancer cells. In response to high ROS level in cancer cells, Nrf2 mediates the transcription of several cytoprotective genes, including those coding for ROS scavenging enzymes, Phase II detoxifying enzymes, drug resistance proteins, drug efflux transporters, and

other stress response molecules. The induction of these genes therefore provides avenue for survival to cancer cells. A lot of evidence has connected aberrant activation of Nrf2 to development of therapeutic resistance and recurrence in different cancers including breast, colorectal, lung, leukemia, liver, pancreatic and ovarian cancer.<sup>15</sup> Stable overexpression of Nrf2, as well as Nrf2 activation by tert-butylhydroquinone increases the resistance of human breast adenocarcinoma and neuroblastoma cells to doxorubicin, etoposide and cisplatin.<sup>14,142</sup> Data from same study also revealed that genetic knockdown of Nrf2 sensitizes lung carcinoma cells to all three chemotherapeutic drugs by repressing the mRNA expression of NQO1 and glutathione.<sup>142</sup> Studies in human doxorubicin-resistant ovarian carcinoma cells indicated an elevated Nrf2 level compared with wild-type control cells.<sup>143</sup> Similarly, other studies reported that cisplatin- and carboplatin-resistant lung carcinoma cells were also characterized by upregulated Nrf2 level.<sup>115,144</sup>

A key mechanism through which Nrf2 drives resistance to chemotherapy and radiation therapy in cancer cells is via upregulation of endogenous antioxidants. Emerging evidence in most cancers points to the induction of antioxidant defense as having a role to play in resistance to most cancer therapies. Cancer cells are believed to endure persistent oxidative stress, a condition thought to contribute to tumor progression. Several studies have demonstrated that cancer cells display higher levels of ROS relative to normal cells.<sup>145–148</sup> The heightened ROS levels, and consequently, the consistently elevated oxidative stress in cancer cells, explain the presence of an elaborate and sophisticated intracellular antioxidant network. This characteristic has led many to hypothesize that it could enhance the resistance of cancer cells to chemotherapy and radiotherapy. Induction of Nrf2 and resultant expression of downstream antioxidant proteins has been associated with resistance to chemotherapy and radiotherapy. In human colorectal cancer cells, it is reported that activation of Nrf2 and subsequent stimulation of thioredoxin reductase 1 (TRX1) reduces ROS generation and drive resistance to 5 fluorouracil.<sup>149</sup> Studies on NSCLC and small cell lung carcinoma (SCLC) cells revealed that increasing resistance to cisplatin is due to decreased platinum-DNA binding and intracellular platinum accumulation driven by elevated levels of antioxidants.<sup>150–152</sup> In addition, Kim et al<sup>153</sup> reported that persistent activation of the Nrf2/ARE pathway is the driver for the enhanced expression of antioxidant proteins such as GCL, HO-1, thioredoxin and peroxiredoxin 1 observed in tamoxifen-resistant breast cancer cells. Furthermore, resistance to 5-FU and  $\gamma$ -irradiation in esophageal squamous carcinoma was attributed to an upregulation of GCLC and GSR caused by a gain of function mutation in Nrf2.<sup>154</sup> Other studies have shown that NQO1 an important downstream target of Nrf2 was upregulated between 2-3-fold in chemoresistant lung, colon, pancreas, and breast cancers.<sup>15,155–158</sup> The role and contribution of another Nrf2 downstream target GSTP1 to chemoresistance was highlighted in breast cancer where its overexpression is reported to be an unfavorable marker for aggressiveness and poor prognosis.<sup>159,160</sup> Further evidence revealed that the expression of GPx1 and GPx2, both members of the glutathione peroxidase (GPx) family were upregulated in cisplatin-resistant NSCLC and A549 cell lines, respectively.<sup>161,162</sup> Evidence also showed that resistance to gemcitabine in pancreatic ductal adenocarcinoma (PDAC) can be attributed to a reduction in ROS generation due to antioxidant expression, especially increase in isocitrate dehydrogenase 1 (IDH1) expression.<sup>163</sup> Similar to these findings, resistance to gemcitabine in pancreatic cancer cells was reported to be due to inhibition of basal and gemcitabine-induced ROS formation by overexpression of SOD2 and CAT, both ROS-scavenging enzymes.<sup>164</sup> Conversely, several studies have shown that genetic or pharmacological inhibition of Nrf2 was able to repress Nrf2-mediated antioxidant gene expression, and thus increase the sensitivity of different tumors to chemotherapeutic drugs and radiotherapy.<sup>66</sup> Inhibition of Nrf2 by siRNA repressed the expression of GSH, thioredoxin 1, peroxiredoxin 1 and NQO1, thus increasing the sensitivity of chemoresistant ovarian cancer cells to cisplatin action.<sup>165</sup> Similarly in resistant prostate cancer cells, reduction in GSH content due to Nrf2 genetic inhibition enhances the vulnerability of the cells to chemotherapy and radiotherapy.<sup>166</sup> Also enhanced sensitization to doxorubicin was observed in human NSCLC A549 cells when GSH level was down-regulated as a result of pharmacological inhibition of Nrf2 by luteolin.<sup>167</sup>

Another mechanism by which Nrf2 facilitates resistance to conventional therapies is via its crucial role in upregulating the expression of genes that encode drug efflux transporters. Constitutive activation of Nrf2 mediates an overexpression of various drug transport proteins such as multidrug resistance protein 1 (MDR1/ABCB1), multidrug resistance-associated proteins (MRPs/ABCCs), and breast cancer resistance protein (BCRP/ABCG2) across different tumor types.<sup>14,15,18</sup> These drug efflux pumps are members of the ATP-binding cassette (ABC) membrane transporter superfamily, and their upregulation decreases chemosynthetic drug accumulation in tumor cells, thus they are associated

with chemoresistance in a number of tumors.<sup>14,15,158</sup> Constitutive induction of ABCC3, ABCG2 and P-glycoprotein are associated with poor prognosis characterized by poor or incomplete response, disease-free response and overall survival in adults with acute myeloid leukemia.<sup>168,169</sup> Evidence has shown that ABCC1 was overexpressed in CUL3-silenced breast cancers and this confers resistance to paclitaxel and doxorubicin.<sup>18,170</sup> Aberrant expression of ABCC1 resulting from Nrf2 activation promotes the efflux of doxorubicin leading to about 2-fold decrease in doxorubicin cytotoxicity in ovarian cancer, leukemia and NSCLC cells.<sup>143,171,172</sup> Resistance to sorafenib-induced apoptosis in hepatocellular carcinoma (HCC) was reported to be associated with Nrf2-induced upregulation of ABCC1, ABCC2 and MDR1.<sup>173</sup> A recent study showed that in tumor tissues from glioma patients, expression of Nrf2 correlates positively with ABCC1 (MRP1) expression and this is associated with tumor aggressiveness, chemoresistance and poor overall survival.<sup>174</sup> Similarly, Nrf2-driven induction of ABCC2 has been implicated in tamoxifen-resistant breast cancer cells and in different cisplatin-resistant human cancer cells.<sup>175–177</sup> Nrf2-mediated upregulation of other ABCs, including ABCC3, ABCC4 and ABCC5 was reported to be the cause of intrinsic resistance to different anticancer drugs (including cisplatin, doxorubicin, etoposide and vincristine) in NSCLC, gastric cancer and hepatocarcinoma cells.<sup>172,177–179</sup> ABCG2 (BCRP) is an ATP hydrolysis (energy)-dependent membrane transporter, and its expression is regulated by Nrf2 binding with ARE area of its promoter region.<sup>15</sup> Some studies revealed an upregulated expression of ABCG2 (between 3–4-fold) in cancer cells with elevated expression of Nrf2. This aberrant expression of ABCG2 was reported to contribute to chemoresistance in those cancer cells.<sup>14,180</sup> Strong correlation between ABCG2 expression and resistance to 5-FU and irinotecan has been reported in breast and colorectal cancer, respectively.<sup>181</sup> In addition, ABCG2 was reported to be overexpressed in BRAF (V600E) mutant A375 melanoma cells and this confers acquired resistance to vemurafenib.<sup>182</sup> Other evidence for the role of ABCG2 in chemoresistance comes from genetic inhibition studies. Nrf2-silenced lung cancer cells showed a down-regulated ABCG2 expression which enhanced their sensitivity to mitoxantrone and topotecan.<sup>183</sup> Furthermore, a recent study revealed that the knockdown of Nrf2 repressed ABCG2 expression and reversed the resistance of PDAC cells to 5-fluorouracil.<sup>184</sup>

Beyond adaptation due to upregulation of endogenous antioxidants and expression of drug efflux transporters, Nrf2 mediates resistance to chemotherapy and other conventional treatments in tumor cells via other mechanisms. The expression of many components of the autophagic system, a known pro-resistance process in tumor cells, is directly regulated by Nrf2. Evidence has shown that Nrf2 induces autophagy via upregulation of p62 with eventual reduction of ROS, and apoptosis inhibition in some tumor cells.<sup>185,186</sup> In addition, promotion of angiogenesis and enhancing cancer stem cells (CSC) self-renewal are other mechanism by which Nrf2 promotes resistance to cancer therapies.<sup>14,18</sup>

Interestingly, the bi-directional mode of action of Nrf2 in tumorigenesis has brought into a clear perspective the importance of the crosstalk between its onco-suppressive and oncogenic roles. This is an indication that maintaining Nrf2 at its optimal level is vital for implementing effective anticancer strategies. The findings from the studies reviewed above have shown that this bidirectional mode of action may be influenced by the genetic makeup of negative regulators of Nrf2 such as KEAP1, as well as the functional crosstalk between Nrf2 and other signaling pathways including P13K/Akt/mTOR, GSK-3 $\beta$ , KRAS/BRAF/c-MYC, p53, NOTCH, STAT3 and NF- $\kappa$ B. The tumor-suppressor role of Nrf2 and its aberrant activation in promoting oncogenesis and mediating therapeutic resistance in different cancer types is complex, and it is a subject that has been addressed in detail in many excellent review articles available in literature.<sup>13–18,62</sup>

## Nrf2, Cytoprotective Proteins and Resistance to Photodynamic Therapy in Cancers

Because of the multi-target mode of action of the PDT process, it is previously believed that the possibility of treated cells to develop protective strategy will be minimal. Thus, PDT is generally assumed to have a low probability of selecting for photo-resistant strain cells. However, more recent data are suggesting that PDT might also induce the cytoprotective mechanisms used by cancer cells to avoid the cytotoxic effect of traditional therapies. Generally, cancer cells are constitutively able to sustain ROS level in the non-toxic range due to the fact that the high ROS level co-evolves with a well-developed and efficient detoxifying antioxidant system. Consequently, this resilient antioxidant system endows tumor cells with high adaptability to oxidative stress-based therapies, including PDT. Although report on therapeutic resistance and the activated survival mechanisms in tumor cells after PDT is very limited, Broekgaarden et al<sup>30</sup> postulated that the survival of tumor cells post-PDT involves a number of interlinked survival pathways, of which

antioxidant response mediated by the Nrf2 transcription factor is one. It is a well-known fact that ROS activates the Nrf2 pathway; however, reports on PDT activation of Nrf2 in tumors are limited. Some studies have reported on the activation of Nrf2 following PDT using different photosensitizer in certain tumors. Activation and translocation of Nrf2 into the nucleus was observed in T24 human bladder and HeLa cells following hypericin-mediated PDT,<sup>187</sup> as observed by us in pigmented MEL 1 and unpigmented A375 melanoma cells.<sup>188</sup> Additionally, Nrf2 downstream target genes including SOD, GCLc, GCLm, NQO 1 and HO-1 have been reported to be upregulated in many cancers after PDT.<sup>51,189,190</sup> We also observed similar upregulation in mRNA expression and activity of several Nrf2 downstream targets including SOD 1, SOD 2, GPx, Trx 1, CAT and GCLc after hypericin-mediated PDT (HYP-PDT) in melanoma cells.<sup>188</sup> The antagonistic nature of antioxidant defense mechanisms to PDT was further highlighted in other studies. Human breast carcinoma MCF-7 cells transfected with GPx gene were shielded from PDT-induced damage after exposure to singlet oxygen.<sup>191</sup> Furthermore, recent studies demonstrated the heterogeneity in the cellular response to 5-ALA-PDT and identified GPx 4 as a possible biomarker and an effector of PDT resistance in breast cancer and glioblastoma cell lines.<sup>192,193</sup> In addition, HYP-PDT-induced cytotoxicity in human embryonic kidney (HEK)-293 cells over-expressing GSTP1 was reported to be diminished compared to wild-type HEK-293 cells.<sup>194</sup> These findings suggest that the upregulation of these genes is cytoprotective and may confer resistance to cancer cells after PDT.

The ability of many tumors to exploit the Nrf2-KEAP 1 pathway for survival and resistance to therapies, including PDT suggest that inhibition strategies for Nrf2 and its targets might attenuate these effects. Nrf2 knockdown increased ROS level and decreased HO-1 expression and thus increased the sensitivity of human ovarian cancer cells to methyl pyropheophorbide a (mPPa)-mediated PDT.<sup>195</sup> Also, Choi et al<sup>196</sup> reported that as a result of Nrf2 knockdown in multiple cancer cells, production of singlet oxygen and other ROS was increased leading to enhanced pheophorbide a (Pba)-PDT-mediated cytotoxicity and increased apoptotic/necrotic cell death of the cancer cells. Furthermore, data from a recent study revealed that pharmacological inhibition of Nrf2 by luteolin sensitizes human pancreatic and bladder T24 cancer cells to porphyrin TMPyP4-mediated PDT. Results from the study indicated that inhibition of Nrf2 by luteolin generates high ROS levels and favors apoptosis by upregulating RKIP expression, while SNAIL expression was downregulated.<sup>197</sup> The takeaway here is that when Nrf2 activity is inhibited, PDT efficacy is enhanced, suggesting a central role for Nrf2 in PDT resistance in cancers.

Specific inhibition of downstream targets of Nrf2 has also been used to shed light on the crucial role of Nrf2-mediated antioxidant response as a possible mechanism of resistance to PDT. Kimani et al<sup>198</sup> reported that different antioxidant inhibitors of the glutathione redox system and ROS scavenging enzymes potentiate the cytotoxicity of disulfonated aluminium-phthalocyanine mediated PDT in breast cancer cells. Similarly, inhibition of glutathione by buthionine sulfoximine (BSO) and carnosine enhanced the sensitivity of human breast adenocarcinoma cell lines, MDA-MB-231 and MCF7, respectively, to hypericin-mediated PDT.<sup>199</sup> A new approach to reduce GSH in tumor tissues relieves hypoxia and enhances the ROS killing effect of PDT by employing multifunctional nanosystem carriers that utilize meta-elements such as copper (Cu), manganese (Mn), iron (Fe) and gold (Au) has been reported to be effective in enhancing tumor cell destruction by PDT.<sup>200–203</sup> HO-1 is a downstream target of Nrf2 which has been closely associated with tumor cell survival post-PDT. The addition of an anti-HO-1 siRNA to WM541Lu human melanoma cells, 24 h prior to PDT increases the responsiveness of the melanoma cells to ALA-based PDT.<sup>204</sup> Zinc(II) protoporphyrin IX, an inhibitor of HO-1 significantly enhanced PDT-mediated cytotoxicity towards human ovarian carcinoma cells.<sup>190</sup> Another study employing siRNA knockdown of HO-1 showed an increased susceptibility of UM-UC-3 human urothelial cell line to ALA-mediated PDT.<sup>205</sup> Further evidence was provided by Kimakova et al,<sup>206</sup> when they demonstrated that resistance to HYP-PDT induced by SOD 2 over-expression in MCF-7 cells was abrogated when the cells were pre-treated with 2-methoxyestradiol (2-ME). Their data revealed that 2-ME increased the sensitivity of the resistant MCF-7 breast cancer cells by decreasing the clonogenic ability of the cells and promoting their apoptosis. Similar improved potentiation of antitumor effects of PDT was observed in other previous studies after genetic or pharmacological inhibition of SOD 2 by 2-ME in neurons, fibroblasts or Jurkat cells.<sup>189,207,208</sup> These reports demonstrate that inhibition of tumor antioxidant system represent a useful approach to enhance the efficacy of photodynamic therapy in different cancers.

Similar to what has been reported during the development of resistance to chemotherapy and other conventional treatments, the expression of drug efflux transporters has been identified as a vital mechanism that can drive tumor

survival post-PDT. Efflux proteins such as P-gp, ABCG2 and ABCCs are overexpressed in majority of cancers. They are known to excrete therapeutic drugs and prevent their accumulation in tumor cells favoring resistance of tumors to different treatment protocols. In the context of PDT, ABCG2 is the most studied drug efflux transporter. It has been implicated in mediating excretion of most photosensitizers, and thus enhances the survival of tumor cells.<sup>30,209</sup> Reports have shown that ABCG2 is involved in the efflux of a number of PS including protoporphyrin IX, hematoporphyrin IX, chlorin e6, pheophorbide a, pyropheophorbide a methyl ester, hypericin and aminoacridine.<sup>209,210</sup>

The importance of the Nrf2-ABCG2 axis in response to PDT-induced ROS generation, restoration of redox homeostasis and induction of tumor survival post-PDT has been highlighted in a number of studies. Overexpression of ABCG2 due to photoactivation of porphyrins in HepG2 cells was downregulated by Nrf2 siRNA knockdown suggesting that Nrf2 is a transcriptional inducer of ABCG2.<sup>88,211</sup> Similar inhibition of ABCG2 expression coupled with increased intracellular Pba level and enhanced cytotoxicity was observed after Nrf2 knockdown in breast cancer MDA-MB-231 and HT-29 cells subjected to Pba-mediated PDT.<sup>196</sup> Further evidence revealed that increasing ROS generation by inhibiting the Nrf2-ABCG2 axis enhanced the sensitivity of human ovarian cancer cells to mPPa-mediated PDT.<sup>195</sup>

Many photosensitizers are known substrates of ABCG2, with evidence showing that ABCG2 affects the pharmacokinetics of these photosensitizers and thus, mediates their efflux. Therefore, many studies have reported on inhibition of ABCG2 to block the efflux of photosensitizers and enhance the sensitivity of tumor cells to PDT. One of the earliest ABCG2 inhibitor used in PDT is the protein tyrosine kinase inhibitor (TKI), Imatinib mesylate. According to results from Liu et al,<sup>212</sup> Imatinib treatment increased the uptake of 2-(1-hexyloxyethyl)-2-devinyl pyropheophorbide a (HPPa, Photochlor), PpIX, and benzoporphyrin derivative monoacid ring A (BPD-MA, Verteporfin) and enhanced the efficacy of PDT in murine radiation induced fibrosarcoma 1 (RIF-1)-ABCG2+ cells and in mice bearing subcutaneous RIF-1 tumors. Gefitinib, another TKI, inhibited ABCG2-mediated efflux of protoporphyrin IX and thus enhanced the efficacy of ALA-PDT in four different malignant brain tumor cells.<sup>213</sup> Kim et al<sup>214</sup> reported an enhancement of pyropheophorbide a (PPa)-mediated PDT in colon cancer cells when ABCG2 was pharmacologically inhibited. Results from their study revealed that in HT-29 (ABCG2 high) and SW480 (ABCG2 low) colon cancer cell lines, expression of ABCG2 was negatively correlated with treatment efficacy. Inhibition of ABCG2 using Ko-143 enhanced the sensitivity of HT-29 cells by blocking PPa efflux and increasing singlet oxygen formation. These results were confirmed in vivo when they demonstrated that Ko-143 pre-treatment to HT-29 cells xenografted nude mice enhanced the sensitivity to PPa-PDT.<sup>214</sup> Similarly, doxorubicin-resistant A270 ovarian cancer cells overexpressing ABCG2 were reported to be more resistant to PPa-PDT compared to non-resistant wild-type ovarian cancer cells.<sup>215</sup> Further data from the study revealed that activation of the c-MET/PI3K signaling pathway is likely associated with doxorubicin resistance. Subsequently, repression of the c-MET and PI3K/Akt pathway, as well as Ko-143 pre-treatment result in down-regulated ABCG2 expression, increased PPa accumulation, increased ROS generation and enhanced cytotoxicity of PPa-PDT to doxorubicin-resistant cells.

Hypericin is a substrate for ABCG2, and the strong affinity of ABCG2 for hypericin was first confirmed by Jendzelovsky et al,<sup>216</sup> when they observed an over-expression of ABCG2 in HT29 cells treated with hypericin. Pre-treated of HT29 cells with an ABCG2 inhibitor fumitremorgin C (FTC), revealed an enhanced accumulation of hypericin in the cells. Subsequent treatment of the HT29 cells with proadifen represses ABCG2 activity and enhances HYP-PDT induced oxidative stress and apoptosis of the HT29 cells. A follow-up study by the same group showed that ABCG2 over-expressing promyelocytic leukemia (HL60) cells showed increased ability to efflux hypericin and are more resistant to HYP-PDT. Ko-143 treatment block ABCG2 over-expression increased hypericin accumulation and enhanced the sensitivity of HL60 cells to HYP-PDT-induced apoptosis.<sup>217</sup> Similarly, Khot et al<sup>218</sup> reported an overexpression of ABCG2 in HYP-mediated PDT-resistant 3D spheroidal modes of HT29 and HCT116 colorectal cancer cell lines compared to their respective 2D cell cultures. Furthermore, inhibiting ABCG2 activity as a result of co-treatment with Ko-143 enhanced the efficacy of HYP-PDT in the 3D spheroidal model of both cancer cell lines.<sup>218</sup> Combining HYP-PDT with inhibition of the 5-lipoxygenase (5-LOX) pathway (co-treatment with 5-LOX inhibitor, MK-886) was reported to decrease ABCG2 protein expression, increase intracellular accumulation of hypericin and improve the sensitivity of HT29 and MCF7 cancer cells to HYP-PDT.<sup>219</sup> Interestingly, a report from the laboratory of some of our collaborators indicated that HYP-PDT displayed differential ABCG2 expression in UCT Mel-1 (ABCG2 upregulation) and A375 (ABCG2 downregulation) melanoma cells. The observed increase in the expression of ABCG2 in A375 cells, however,

did not translate to increased resistance, suggesting that ABCG2 expression may not influence HYP-PDT in those cell lines.<sup>220</sup> Overall, the studies reviewed here indicated that inhibition of ABCG2 to block the efflux of photosensitizers has emerged as a promising strategy to enhance the sensitivity of cancer cells to PDT. Inhibition of ABCG2 leads to increased intracellular accumulation of photosensitizers, higher ROS generation, and enhanced cytotoxicity, ultimately improving the effectiveness of PDT in different cancer types.

## Conclusion and Future Perspectives

Photodynamic therapy (PDT) is a treatment modality for cancer that involves the use of photosensitizers, light, and oxygen to generate reactive oxygen species (ROS) that induce tumor cell death. However, the efficacy of PDT can be limited by the development of drug resistance. Nrf2 is a transcription factor that regulates the expression of genes involved in the cellular antioxidant response. Activation of Nrf2 in tumor cells can lead to the upregulation of expression of genes coding for antioxidant enzymes and drug efflux transporters. Antioxidant enzymes such as superoxide dismutase (SOD) and catalase, glutathione peroxidase, and heme oxygenase 1 can scavenge ROS generated by PDT, while drug efflux transporters can pump photosensitizer drugs out of tumor cells, thus limiting their accumulation and efficacy. Several studies reviewed here provide evidence showing that Nrf2 is aberrantly expressed in cancer cells resistant to PDT. Similarly, we reported evidence of overexpression of downstream targets of Nrf2, including various drug efflux transporters in cancer cells that are resistant to PDT. While the molecular mechanism(s) underlying the resistance of tumor cells to PDT may not be fully understood yet, overwhelming evidence has pointed to Nrf2-mediated antioxidant response and drug efflux transporter upregulation as possible mechanisms of resistance in PDT of cancers. To overcome this, strategy to downregulate the expression of Nrf2, including combining PDT with Nrf2 inhibitors to increase the cytotoxicity and efficacy of PDT needed to be vigorously pursued. Also, encapsulation of photosensitizers within nanocarriers in order to enhance their distribution to target tissues will help to protect PS from drug efflux transporters, and thus block a major limitation to PDT efficacy.

## Consent for Publication

The details of images can be published and all authors provide consent for the article contents to be published.

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## Author Contributions

All authors made a significant contribution to the work reported, whether that is in the conception, study design, execution, acquisition of data, analysis and interpretation, or in all these areas; took part in drafting, revising or critically reviewing the article; gave final approval of the version to be published; have agreed on the journal to which the article has been submitted; and agree to be accountable for all aspects of the work.

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