



NAC, NAC, Knockin' on Heaven's door: Interpreting the mechanism of action of *N*-acetylcysteine in tumor and immune cells

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ABSTRACT

N-acetylcysteine (NAC) has been used as a direct scavenger of reactive oxygen species (hydrogen peroxide, in particular) and an antioxidant in cancer biology and immuno-oncology. NAC is the antioxidant drug most frequently employed in studies using tumor cells, immune cells, and preclinical mouse xenografts. Most studies use redox-active fluorescent probes such as dichlorodihydrofluorescein, hydroethidine, mitochondria-targeted hydroethidine, and proprietary kit-based probes (i.e., CellROX Green and CellROX Red) for intracellular detection of superoxide or hydrogen peroxide. Inhibition of fluorescence by NAC was used as a key experimental observation to support the formation of reactive oxygen species and redox mechanisms proposed for ferroptosis, tumor metastasis, and redox signaling in the tumor microenvironment. Reactive oxygen species such as superoxide and hydrogen peroxide stimulate or abrogate tumor cells and immune cells depending on multiple factors. Understanding the mechanism of antioxidants is crucial for interpretation of the results. Because neither NAC nor the fluorescent probes indicated above react directly with hydrogen peroxide, it is critically important to reinterpret the results to advance our understanding of the mechanism of action of NAC and shed additional mechanistic insight on redox-regulated signaling in tumor biology. To this end, this review is focused on how NAC could affect multiple pathways in cancer cells, including iron signaling, ferroptosis, and the glutathione-dependent antioxidant and redox signaling mechanism, and how NAC could inhibit oxidation of the fluorescent probes through multiple mechanisms.

1. Introduction

N-acetylcysteine (NAC), also referred to as *N*-acetyl-L-cysteine, was first approved by the US Food and Drug Administration in 1963 as a drug to treat excessive mucous production in respiratory diseases including cystic fibrosis [1]. It was later used in treatment for paracetamol (i.e., acetaminophen or Tylenol) poisoning [1]. NAC has been used as a Von Willebrand factor modifying agent to mitigate the development of arterial thrombosis [2]. NAC, a membrane-permeable cysteine precursor, is used as a reactive oxygen species (ROS) scavenger and a potent antioxidant [3]. Its cytoprotective effect and/or inhibition of oxidation of fluorescent dyes was related to its ability to scavenge ROS (superoxide or hydrogen peroxide [H_2O_2]) and modulate redox signaling effects in cancer cells [4]. However, neither superoxide nor H_2O_2 reacts at an appreciable rate with NAC [5,6]. This calls into question the ROS scavenging as a direct antioxidant mechanism of NAC. NAC can enhance the intracellular cysteine pool; release protein thiols through disulfide cleavage, thus increasing glutathione (GSH) levels; and enhance the activity of GSH-dependent detoxification of H_2O_2 by antioxidant

enzymes (glutathione peroxidase, thioredoxin) [7–9]. GSH is a direct antioxidant, being a substrate for antioxidant enzymes (glutathione peroxidase). Thus, the proposed antioxidant mechanism of NAC is not related to the direct scavenging of ROS (superoxide or H_2O_2) or a direct antioxidant mechanism, acting as a substrate for antioxidant enzymes or even as a direct scavenger of drug-derived electrophilic metabolites. For example, NAC scavenging of the metabolite, *N*-acetyl-quinoneimine, formed from Tylenol as the major detoxication mechanism, was questioned [10]. The availability of L-cysteine is a rate-limiting factor in GSH biosynthesis, and NAC is used as a precursor of L-cysteine, but the mechanism by which NAC induces GSH synthesis *in vivo* remains to be established. The results from using sulfur-35 radioactive isotope-labeled NAC as a tracer suggest an indirect mechanism for releasing L-cysteine and inducing intracellular GSH and redox status [11]. The effects of NAC may be indirectly related to detoxication of H_2O_2 , enhancement of GSH synthesis, and/or enhancement of nuclear factor erythroid 2-related factor 2 (NRF2) [10]. This is crucial for understanding and advancing redox signaling mechanisms in cancer [12]. Reports also suggest that in mitochondria, NAC undergoes metabolism to hydrogen sulfide and

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different sulfane sulfur species that are more potent scavengers of oxidants [13,14]. Fig. 1 shows the potential direct and indirect pathways by which NAC could intervene in redox-dependent signaling pathways and GSH *de novo* synthesis from cysteine, glutamate, and glycine. How NAC affects their transport mechanisms into cells is unknown. NAC is typically used in high concentrations to elicit significant effects in cell culture and in preclinical studies. A recent consensus publication on ROS detection and antioxidants [6] recommendations clearly specifies the mechanism of action of an antioxidant, the identity of the ROS detected, the efficacy of the reaction between the ROS and antioxidant, and the effect of the antioxidant in cells at the concentrations effective for ROS detoxication [6]. The authors have highlighted the ambiguities associated with using the term “antioxidant” without defining or discussing its potential mechanism of action [6]. In numerous cancer biology studies, the effects of NAC have been attributed to its role as a radical scavenging antioxidant. A recent report cautioned against the use of random antioxidant therapy due to the complexity of ROS regulation in cancer progression [15]. Clearly, determining the identity of ROS is critical before implementing an antioxidant therapy in diseases.

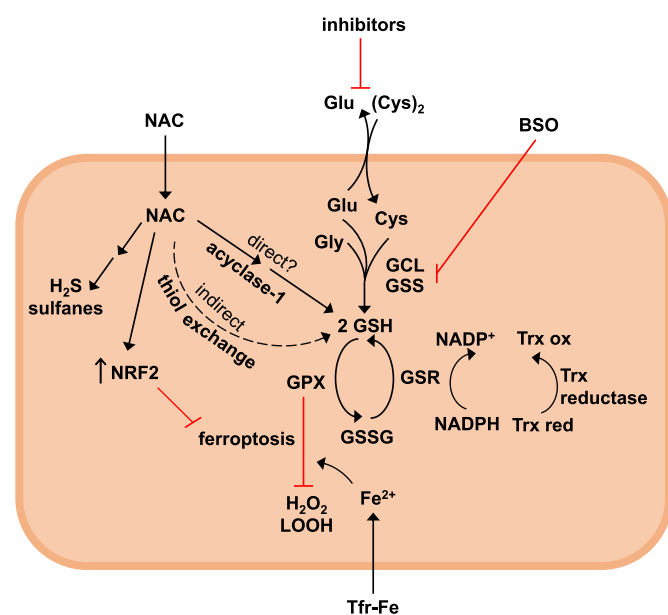


Fig. 1. Redox-dependent antioxidant mechanisms of NAC. Intracellular GSH is synthesized from three amino acids: cysteine, glutamine, and glycine. Glutamine and cysteine levels are increased through transport of extracellular cystine and glutamine imported into cells. Glutamate-cysteine ligase (GCL) enables formation of γ -glutamyl cysteine and glycine catalyzed by GCL and glutathione synthetase (GSS) enzymes. GSH is formed by the GSS-catalyzed reaction between γ -glutamyl cysteine and glycine. Redox coupling to NADPH/NADP⁺ via thioredoxin (Trx) reductase-mediated oxidation/reduction of thioredoxin regulates the GSH/oxidized glutathione (GSSG) pathway catalyzed by glutathione reductase (GSR). GPX detoxifies H₂O₂ and lipid hydroperoxide using GSH as a substrate. This pathway also regulates transferrin receptor mediated iron signaling. NAC enhances GSH levels through a direct or indirect mechanism enhancing the detoxication of H₂O₂ and lipid peroxides described above. NAC also inhibits iron/heme catalyzed oxidations (ferroptosis), preventing the formation of more potent oxidants. NAC stimulates the NRF2-regulated cellular free iron responsible for iron-mediated lipid peroxidation or ferroptosis. NAC stimulates NRF2, thus inhibiting ferroptosis. NAC is metabolized to sulfanes that can directly react with ROS (superoxide and H₂O₂) and decrease their intracellular levels. This is an oversimplified version of the redox-coupled processes involved in the H₂O₂ detoxifying pathway. It does not include a redox relay for H₂O₂ signaling through the redox activation pathway involving peroxiredoxin, STAT3 (signal transducer and activator of transcription 3), and thioredoxin [122]. However, NAC can influence ROS mechanisms through redox regulation of thiol-containing redox couples.

The effects of ROS (superoxide and H₂O₂) in cancer are shown to be paradoxical [5,16]. At low levels, superoxide/H₂O₂ induce cell proliferation and promote tumorigenesis and progression, and at higher levels they are cytotoxic to cancer cells and inhibit metastasis. Thus, inhibiting ROS could mitigate tumorigenesis and progression while stimulating metastasis. Antioxidants that directly or indirectly remove ROS affect tumorigenesis, progression, and metastasis of tumor cells differently. NAC was shown to exert paradoxical effects in a mouse lung cancer model [17,18]. NAC treatment enhanced metastasis of lung cancer [16, 17]. This could be due to decreased oxidative stress in metastatic tumors, causing them to proliferate and grow in distant sites [17]. Pre-treatment of melanoma cells with NAC prior to intravenous injection in mice enhanced tumor formation by tenfold [19]. The findings from the mouse melanoma progression model predict that the use of dietary antioxidants or antioxidant therapy might protect against the deleterious effects of oxidants in metastatic melanoma cells, and that the antioxidant therapy is a contraindication for melanoma [19].

Elevated levels of GSH promote metastasis in both melanoma and liver cancer [20,21]. In addition, NAC administration promotes distant metastasis [17]. This means that elevated levels of GSH in tumor cells intensify cancer progression. NAC treatment enhanced the *ex vivo* expansion of single circulating tumor cells comprising a small set of metastatic precursors through mitigation of oxidative stress [22]. NAC sensitizes pancreatic cancer cells to gemcitabine [23]. Reports emphasize the dual roles of ROS and GSH in cancer initiation and progression [24,25]. Potential beneficial effects of NAC treatment in triple-negative breast cancer were attributed to mitigation of ROS in the tumor microenvironment [26]. The mechanism by which NAC decreased ROS or the identity of ROS was not specified. It was reported that the combined administration of interleukin-2 and NAC synergistically enhanced the intracellular levels of GSH while enhancing the cytotoxicity of interleukin-2/lymphokine-activation therapy [27]. *In vivo* supplementation of NAC inhibited proliferation of breast cancer cells [28]. NAC inhibits stromal cell metabolism and oxidative stress, preventing the increased uptake of lactate by breast cancer cells [28]. Reports also suggest that NAC could prevent proliferation, migration, and invasion in an antioxidant-independent manner via targeting Notch2 signaling in glioblastoma multiforme [29].

Oxidative modifications of phosphatases that possess an active-site cysteine residue involve oxidation of the thiolate anion to sulfenate, sulfinate, or sulfonate states depending on the H₂O₂ concentration and duration of exposure. This results in their inactivation and an increase in mitogen-activated protein kinases (MAPK) and protein kinase B activity, leading to increased cell proliferation. Thus, mitigation of oxidant-induced effects, restoration of the phosphatase activity, or suppression of MAPK and protein kinase B activity will inhibit cell proliferation and survival. NAC-mediated attenuation of lung adenocarcinoma in an oncogenic KRAS G12D-driven mouse model (with increased NADPH oxidase 2 and decreased NRF2) was attributed to pharmacological suppression of ROS by NAC [30].

NAC enhanced the antitumor effect of chimeric antigen receptor (CAR)-modified natural killer cells [31]. Also, NAC stimulated the antitumor function of the exhausted T cells. The proposed mechanisms for the stimulatory effects of NAC in immune cells are similar to those suggested in cancer cells.

In this review, I discuss and critically evaluate the assays and probes, as well as the results derived therefrom, to assess NAC-mediated radical scavenging antioxidant mechanisms and immunomodulatory effects in cancer biology and immunotherapy.

2. H₂O₂ does not directly react with the DCFH redox-active probe

The assay based on dichlorodihydrofluorescein (DCFH) oxidation to 2',7'-dichlorofluorescein (DCF) has been used to measure intracellular H₂O₂ [32]. Inhibition by DCF fluorescence in NAC-treated cells was

viewed as an absolute proof for H_2O_2 formation in cancer cells. However, neither NAC nor DCFH reacts with superoxide or H_2O_2 at an appreciable rate [32–34]. Yet, the green fluorescence due to DCF in endothelial cells was inhibited by NAC treatment [32]. Iron chelators or GSH modulators inhibited intracellular DCFH oxidation to DCF [32].

It was reported that phenyl isothiocyanate-induced ROS in cancer cells measured by DCF fluorescence was reversed by NAC [35]. Other mechanisms of NAC were not considered. On a cautionary note regarding the use of NAC as an antagonist to assess isothiocyanate-induced ROS-mediated apoptosis, the investigators indicated that NAC pretreatment significantly inhibited the cellular uptake of isothiocyanates by conjugating with them in the medium [36]. Supplementation of different mouse models of cancer with exogenous thiol-containing antioxidants (such as NAC or GSH) promoted tumor formation and growth [37]. The pro-oncogenic effects of NAC and GSH were attributed to neutralizing ROS and protecting the cancer cells from ROS-induced damage. ROS levels were monitored using DCF fluorescence. As discussed above, changes in DCF fluorescence are due to multiple factors and do not directly measure ROS [32].

3. Oxidant-induced iron signaling: increased oxidation of DCFH

Previous studies implicated a role for redox-active iron in H_2O_2 -induced intracellular oxidation of DCFH to a green fluorescent product, DCF [32]. We used this assay—oxidation of DCFH to DCF—to monitor transferrin receptor (TfR)-mediated iron signaling induced by mitochondrial complex I inhibition and inactivation of cytosolic aconitase. The iron levels in cells are regulated by the cell surface TfR-mediated uptake of iron as transferrin iron [38]. TfR synthesis is regulated by the interaction of the iron regulatory protein (IRP) with the iron-responsive element present in the 3'-untranslated region of TfR mRNA. IRPs serve as sensors of cellular iron [38]. A major portion of cellular iron is utilized for the assembly of iron-sulfur clusters and heme biosynthesis in mitochondria. Inactivation of mitochondrial electron transport chain complexes and/or inactivation of the [4Fe–4S] cluster in mitochondrial aconitase by intracellular oxidants is sufficient to stimulate cellular iron sulfur signaling [32]. Inactivation of aconitase and subsequent activation of IRPs act as sensors of cellular iron status. The IRPs bind with a high affinity to the iron-responsive element present on TfR and ferritin mRNAs. The increased binding to TfR mRNA stabilizes the mRNA, leading to enhanced mRNA translation and increased TfR synthesis. Previous reports suggest a potential link between oxidant-induced iron signaling and DCFH oxidation. Results obtained with an anti-TfR antibody strongly suggest that iron transported into cells via TfR is responsible for H_2O_2 -induced DCFH oxidation and that intracellular oxidative stress, caused by GSH depletion, triggers iron signaling and apoptosis [32]. Antioxidants inhibit TfR expression and iron uptake. DCFH is oxidized by either hydroxyl radical or alkoxyl radical generated by an anti-TfR antibody-dependent Fenton mechanism (Fe^{2+} and H_2O_2 or lipid hydroperoxide reaction) or perferryl iron formed by the heme protein and H_2O_2 reaction. We did not realize at that time that a ferroptotic-type signaling mechanism could be involved. Measurement of extracellularly added ^{55}Fe uptake into cells was inhibited by pretreatment with an anti-transferrin antibody [32]. Studies also indicate that DCFH oxidation may be caused by oxidants generated by the interaction between heme proteins (cytochrome *c*) and H_2O_2 [39]. Numerous reports indicate that oxidation of mitochondrial aconitase induces TfR-mediated iron signaling and oxidative stress-mediated regulation of IRPs [32,40–47].

4. Reinterpretation of results from fluorescence assays using HE and NAC

Hydroethidine (HE) was used to detect superoxide by measuring the 2-hydroxyethidium (2-OH-E^+) (Fig. 2). HE is also oxidized to ethidium (E^+) in superoxide-generating systems containing redox metal ions,

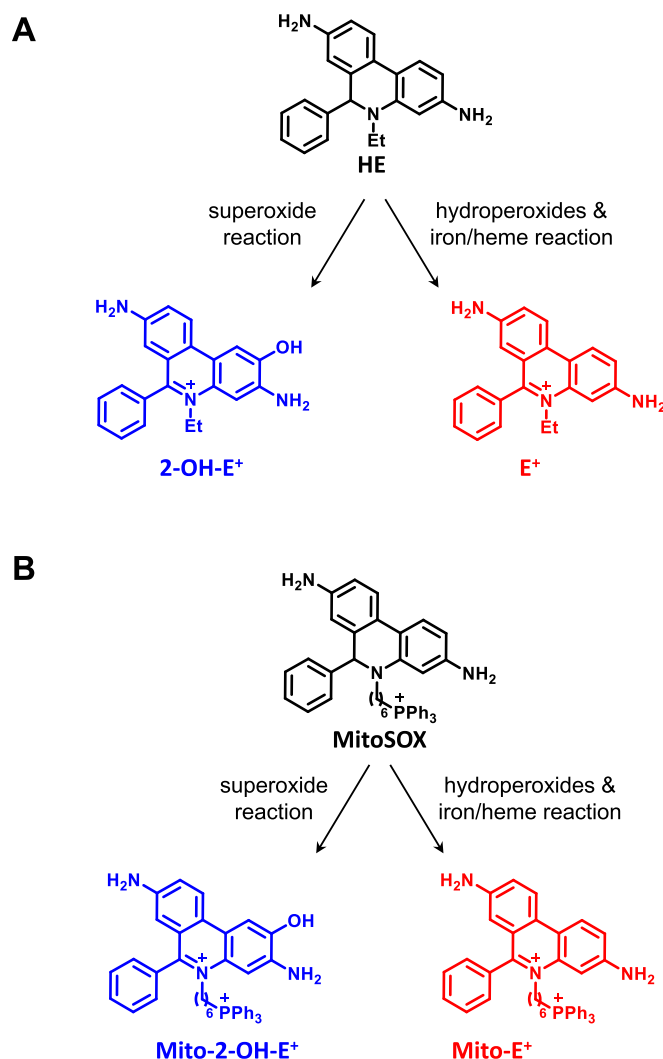


Fig. 2. Superoxide-mediated hydroxylation and oxidation of HE and MitoSOX. Superoxide can directly react with HE or Mito-HE, forming the hydroxylated products that are specific for superoxide (shown in blue). In the intracellular milieu, cellular iron or heme proteins in the presence of H_2O_2 and phospholipid hydroperoxides can oxidize HE and MitoSOX to other oxidation products (shown in red). The fluorescence spectra of 2-OH-E^+ and E^+ as well as Mito-2-OH-E^+ and Mito-E^+ overlap considerably. Advanced spectral imaging techniques could resolve this problem in the future as discussed in the review [123]. Reprinted from Redox Biology, Vol. 15, Balaraman Kalyanaraman, Gang Cheng, Micael Hardy, Olivier Ouari, Brian Bennett, Jacek Zielonka, Teaching the basics of reactive oxygen species and their relevance to cancer biology: Mitochondrial reactive oxygen species detection, redox signaling, and targeted therapies, Pages 347–362, Copyright 2018, with permission from Elsevier. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

hemes, or peroxidases. Because of overlapping fluorescence characteristics between 2-OH-E^+ and E^+ , confocal fluorescence microscopy or flow cytometry cannot be used to measure superoxide, as these techniques predominantly measure E^+ . Investigators assumed that changing the dye from DCFH to HE and monitoring the fluorescence is equivalent to measuring superoxide. Alterations in intracellular thiols, including GSH in NAC-treated cells, which inhibit formation of oxidants, would inhibit HE oxidation as well. We obtained similar results in doxorubicin-treated cells [48]. We attributed the DCFH-derived green fluorescence to a reaction with H_2O_2 and HE-derived red fluorescence to superoxide. That was an incorrect interpretation on our part with respect to HE-derived red fluorescence and intracellular superoxide. As we

described previously [49], redox probes are oxidized by an oxidative mechanism to the corresponding two-electron oxidation products (Fig. 3). The major difference between superoxide and other oxidants with respect to using HE is that 2-OH-E⁺ is the diagnostic marker product of the superoxide/HE reaction.

5. Reinterpretation of fluorescence assay using MitoSOX and NAC

NAC protects against melanocytes, oxidative stress, and UV-induced melanoma in mice. As described, DCFH was used for H₂O₂ and HE for superoxide [50]. HE typically is used as a fluorescent probe that is specific to detecting intracellular superoxide by monitoring the red fluorescence. This is based on the incorrect assumption that superoxide oxidizes HE to E⁺ that has red fluorescence [51]. Although, under conditions generating only superoxide, HE is oxidized to 2-hydroxyethidium and not E⁺, in the presence of iron or perferryl iron, HE is also oxidized to E⁺ in cells. Similarly, using mitochondria-targeted hydroethidine (MitoSOX), *i.e.*, HE conjugated to a triphenylphosphonium

moiety, as a probe, it has been wrongly assumed that superoxide reacts with MitoSOX to generate the red fluorescent product, Mito-E⁺. In the presence of iron and perferryl iron, MitoSOX is also oxidized to the nonspecific Mito-E⁺ responsible for intracellular red fluorescence (Fig. 2). No matter the structure of the redox probes (HE, DCFH, or MitoSOX), intracellular iron or heme peroxidase catalyze the oxidation of HE, DCFH, and MitoSOX in the presence of H₂O₂ to the corresponding fluorescent product (Fig. 3). We can also consider the commercial probe, CellROX Green Reagent, in the same category. However, the actual structure of the probes is unknown, which makes it impossible to interpret the results obtained using these probes. CellROX Deep Red and CellROX Green Reagents, two fluorogenic and cell-permeable probes, were used to measure intracellular ROS. These redox probes are weakly fluorescent in the reduced forms but highly fluorescent in the oxidized forms. Unfortunately, the structures of these probes are proprietary and, therefore, unknown. Based on the information provided by the manufacturers, it is highly likely that these probes share a similar structural chromophore to other probes discussed above, and they are unlikely to react directly with superoxide or H₂O₂ but undergo oxidation in the

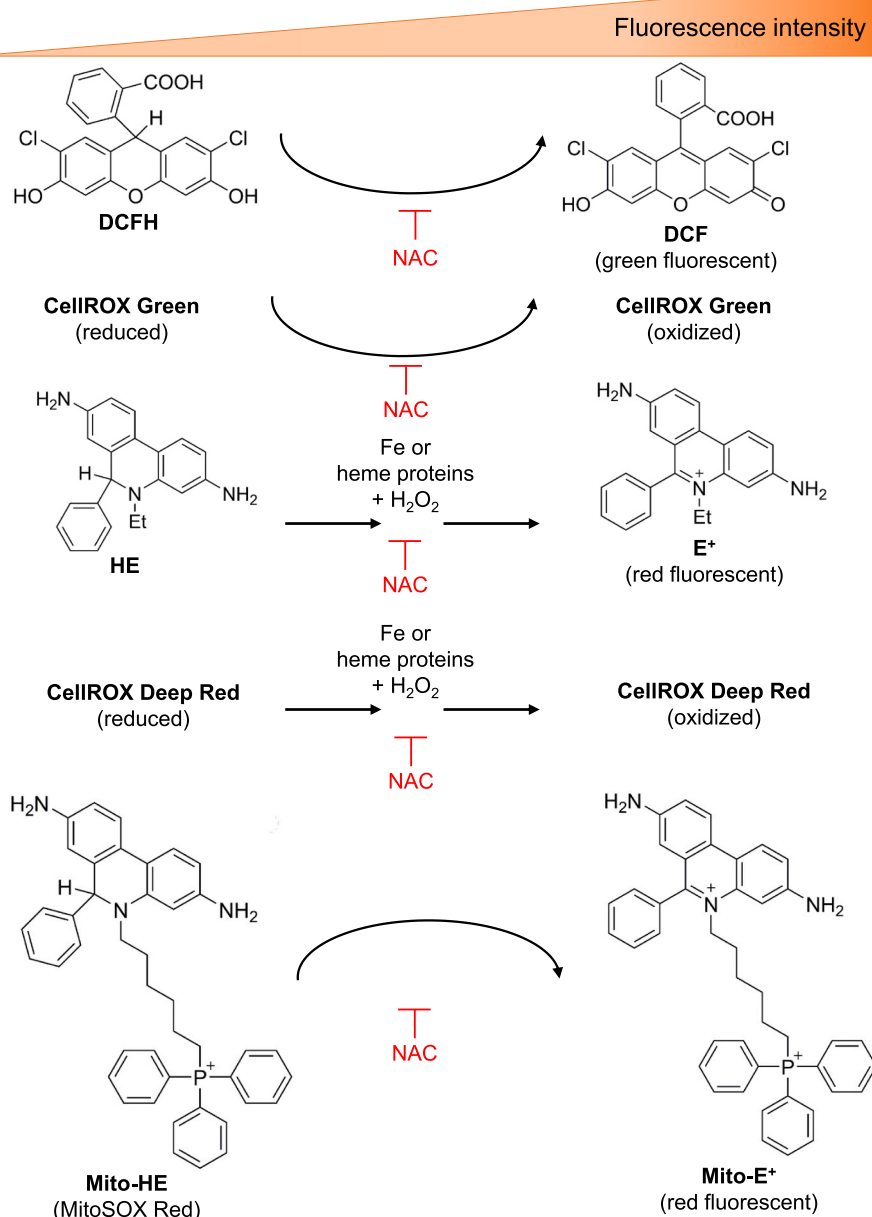


Fig. 3. Intracellular oxidation of selected probes by oxidants generated by H₂O₂ and iron or heme. Different probes, such as DCFH, CellROX Green, HE, CellROX Deep Red, and Mito-HE (MitoSOX Red), are used to detect ROS by monitoring the intracellular fluorescence of their oxidized products, assuming that the oxidation of probes occurs via reaction with superoxide or H₂O₂. NAC treatment inhibits fluorescence derived from different probes. However, neither superoxide nor H₂O₂ alone can oxidize the reduced probes to oxidized products and other mechanisms (ferroptosis or peroxidase activity in the presence of intracellular H₂O₂ and iron or heme/heme proteins) are involved. Thus, oxidants derived from H₂O₂ and iron or heme/heme protein are able to oxidize the different ROS probes to the corresponding oxidation products. Neither superoxide nor H₂O₂ reacts with NAC at an appreciable rate, although NAC has been reported to inhibit intracellular fluorescence from these probes. Modified from Redox Biology, Vol. 15, Balaraman Kalyanaraman, Gang Cheng, Micael Hardy, Olivier Ouari, Brian Bennett, Jacek Zielonka, Teaching the basics of reactive oxygen species and their relevance to cancer biology: Mitochondrial reactive oxygen species detection, redox signaling, and targeted therapies, Pages 347–362, Copyright 2018, with permission from Elsevier. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

presence of H₂O₂ and iron or perferryl iron.

NAC was used to confirm the antioxidant role for TP53-induced glycolysis and apoptosis regulator (TIGAR). Dynamic regulation of TIGAR expression was attributed to both low and high ROS levels during the development of pancreatic ductal adenocarcinoma [52]. Mitochondrial ROS levels were measured by MitoSOX fluorescence [52]. MitoSOX fluorescence was measured by MitoSOX Red, a presumed mitochondrial superoxide indicator in this study [52]. MitoSOX fluorescence was increased in TIGAR knockout pancreatic ductal adenocarcinoma cells as compared with control cells, and NAC treatment decreased MitoSOX fluorescence in TIGAR knockout pancreatic ductal adenocarcinoma cells [52]. Although, the identity of ROS was not specifically mentioned as mitochondrial superoxide, one could infer that MitoSOX fluorescence intensity changes are attributed to changes in mitochondrial superoxide. In another model, cell lines were derived from tumors isolated from control, TIGAR-deleted, and TIGAR-deleted but supplemented with NAC cells. ROS was measured by anti-malondialdehyde (MDA) staining of peroxidized lipids as a marker of oxidative stress. MDA staining was increased in TIGAR-deleted cells, and NAC treatment decreased MDA staining that was attributed to the antioxidant mechanism of NAC. From the discussion above, it is apparent that MitoSOX fluorescence results do not provide experimental proof for increased generation of mitochondrial superoxide in TIGAR-deleted cells, and that the enhanced MitoSOX fluorescence is likely due to enhanced oxidation of MitoSOX to Mito-E⁺ by H₂O₂ and iron or perferryl iron (Fig. 3). Measurement of the hydroxylated product from MitoSOX (Mito-2-OH-E⁺) would be indicative of mitochondrial superoxide (Fig. 2).

The inhibitory effect of NAC in MitoSOX fluorescence in TIGAR-depleted cells is due to decreased formation of oxidants involving peroxide and iron or heme. Because MDA is formed by redox metal iron or heme catalyzed decomposition of lipid hydroperoxides, inhibition of MDA staining in TIGAR-deleted cells treated with NAC is not due to direct scavenging of hydroperoxides.

In another study, NAC was used to support the hypothesis that monocarboxylate transporter 1 inhibition decreased circulating metastatic melanoma cells by enhancing oxidative stress in metastasizing cells but not in primary xenografts [53]. The dye CellROX Deep Red was used to detect ROS. Although the structure of these probes is unknown, based on the fluorescence characteristics, these probes share the structural chromophore of either DCFH or HE. Neither the mechanism of oxidation of the dye nor the antioxidant mechanism of inhibition by NAC is known. As discussed above, it is likely that oxidation of the dye is initiated by redox iron or heme and H₂O₂ (Fig. 3). NAC treatment invariably inhibited oxidation of the fluorescent probe, regardless of its structure (Fig. 3). As direct scavenging of superoxide or H₂O₂ by NAC is unlikely; therefore, the mechanism by which NAC inhibits oxidation of dyes is more complex. Additional investigation into this mechanism is required.

6. NAC inhibition of ferroptosis in cancer cells

Ferroptosis is an iron-dependent intracellular lipid peroxidation resulting in oxidative cell death [54]. Ferroptosis can occur through a variety of stress-related mechanisms, and the two key events triggering ferroptosis are excessive iron accumulation and inhibition of lipid hydroperoxide detoxifying enzymes such as glutathione peroxidase 4 (GPX4). GPX4 is a GSH and selenium-dependent peroxidase that can detoxify phospholipid hydroperoxides to lipid alcohol in concert with other thiol-dependent redox enzymes, so factors or drugs affecting the uptake of cystine and reduction to cysteine could inhibit GPX4 activity and enhance ferroptosis (Fig. 1). Inhibition of GSH levels or GSH synthesis induce intracellular glutamate and ferroptotic cell death.

Lipid peroxidation is free radical-induced oxidation of polyunsaturated fatty acid (PUFA) such as linoleic acid or arachidonic acid containing two or more double bonds. Monounsaturated fatty acids

(MUFA) containing a single double bond are not susceptible to radical-induced lipid peroxidation. This differential susceptibility to peroxidation is the reason why MUFA and PUFA have antagonistic effects in ferroptosis—enrichment with MUFA inhibits ferroptosis and enrichment with PUFA enhances ferroptosis [55]. Enzymatic machineries responsible for synthesis and incorporation of PUFA and MUFA in cell membranes are targets for regulating ferroptosis [56]. GPX4 prevents lipid peroxidation and ferroptosis [57]. Suppression of GPX4 induced ferroptotic iron signaling and activation [57].

NAC may inhibit ferroptosis in cancer cells through activation of NRF2; control of the expression of metallothionein, ferritin, and ferroportin; and inhibition of iron accumulation. Other NRF2 target genes (GPX4, GSH, and NADPH syntheses) and epigenetic regulators responsible for regulating lipid hydroperoxides are also involved in ferroptosis [58–60]. The role of NRF2 in cancer is paradoxical—whether it is a tumor suppressor or tumor promoter—depending upon the cell type, stage of tumorigenesis, and levels of NRF2 expression [61,62]. Targeting the ferroptosis pathway is emerging as a viable therapeutic strategy in cancer treatment and other diseases [63–65].

7. NAC and mitochondrial transfer from stromal cells to cancer cells

Mitochondrial transfer from stromal cells was shown to occur in various cancers including breast cancer, lung cancer, and melanoma [66]. Cancer cells use a mitochondrial stromal transfer mechanism to acquire energy for their growth [66,67]. Investigators have used NAC in mitochondrial transfer studies to glean mechanistic insights. Briefly, mitochondrial transfer involves the incorporation of mitochondrial genes or the whole mitochondria from the host cell (e.g., stromal cells) into a recipient cell (e.g., cancer cells). This transfer can be regulated by extracellular vesicles, tunneling nanotubes, or gap junctions. Readers should refer to the figures in Saha et al. [67] for an illustration of nanotube-mediated mitochondrial transfer from immune cells to cancer cells. Mitochondrial transfer from stromal cells to cancer cells restored mitochondrial activities in cancer cells, including increased ATP generation, membrane potential, and oxygen consumption [66]. ROS generation in cancer cells through activation of the NADPH oxidase enzyme stimulated the mitochondrial transfer from stromal cells to cancer cells [68]. Studies show that tunneling nanotubes facilitate mitochondrial transfer from bone marrow stromal cells (BMSCs) to acute myeloid leukemia (AML) that is regulated by ROS [69,70]. In contrast with most cancers, the survival of AML was suggested to be dependent on mitochondrial oxidative phosphorylation. AML cells increased mitochondrial mass as compared with nonmalignant hematopoietic stem cells. AML-induced ROS was proposed to drive mitochondrial transfer from BMSCs by increasing oxidative stress in the nonmalignant donor cells [68], and were measured using the Amplex Red, DCFH, or CellROX. NAC inhibited mitochondrial transfer. Superoxide was measured using the Amplex Red assay and H₂O₂ by the DCF assay [68]. The rationales for the specific use of these probes in the identification of ROS were not articulated.

NAC treatment inhibited mitochondrial transfer from BMSCs to AML. The implication was that NAC-mediated inhibition of mitochondrial transfer is linked to inhibition of ROS by NAC. It is not clear as to how enhanced oxidation of ROS probes occurred in these cells, nor is it understood as to how NAC inhibits transfer of mitochondria from BMSCs to AML. In another study, ROS-inducing chemotherapeutics-stimulated mitochondrial transfer from stromal cells to acute lymphoblastic leukemia cells was shown to be inhibited by NAC [71]. CellROX Green staining was used to quantitate ROS. The inhibitory effect of NAC on CellROX-derived fluorescence does not equate to its action on superoxide or H₂O₂. Melanoma cells that are typically glycolytic regain oxidative phosphorylation function through the transfer mitochondria from mesenchymal stromal cells [72]. NAC stimulates PGC-1 α in mesenchymal stromal cells. NAC was used as a scavenger of H₂O₂ [72],

as measured by the Amplex Red assay. ROS was suggested to play a critical role in inducing the Akt/PI3K/mTOR signaling pathway during intracellular mitochondrial transfer [73]. NAC-mediated attenuation of mitochondrial transfer was attributed to ROS scavenging [73]. Pathogens, such as lipopolysaccharides, that induced mitochondrial transfer from the bone marrow stem cells to hematopoietic stem cells were inhibited by NAC [74]. ROS scavenging by NAC was implicated [74]. NAC induces quiescent-like pancreatic stellate cells from an active state and attenuates cancer–stroma interactions [75].

In summary, mitochondrial transfer from neighboring cells in the tumor microenvironment to tumor cells can restore and enhance their mitochondrial function, which leads to an enhanced rate of proliferation and enhanced tumor progression and metastasis [66]. The inhibitory effects of NAC in mitochondrial transfer from stromal cells to cancer cells are intriguing; however, the simplistic view (direct scavenging of ROS like H₂O₂ by NAC) impedes an in-depth understanding of thiol-dependent ROS signaling mechanisms (e.g., ref. [76]) in intracellular mitochondrial transfer from stromal cells to cancer cells and the subsequent metabolic reprogramming. More rigorous and advanced analytical and imaging techniques are likely needed to fully understand the redox reactions and mechanisms of NAC.

8. Hyperpolarized MRI studies using NAC

NAC is membrane-permeable and has been reported to cross the blood–brain barrier in humans and rodents, although its bioavailability is low. Recently, investigators reported a state-of-the-art hyperpolarized MRI technique using a hyperpolarized [1-¹³C] NAC probe to monitor the redox status in tumor tissues [77]. Hyperpolarization is a novel and emerging technology that can dramatically enhance the signal-to-noise ratio in magnetic resonance. Briefly, this technique involves imaging of carbon-13 (¹³C)-labeled molecules using MRI. The ¹³C nucleus (magnetic quantum number, I = 1/2) is nuclear magnetic resonance active. However, its natural abundance is only 0.1%. As a result, its MRI signal is low. Although ¹³C enrichment increases the signal, hyperpolarized MRI using ¹³C increases the signal-to-noise ratio by more than 10,000 times in liquid-state nuclear magnetic resonance [78–81]. Hyperpolarization using dynamic polarization enables transient redistribution of the nuclear spin states [82]. Dynamic polarization uses microwave energy to transfer the polarization from unpaired electrons in a trityl radical to the ¹³C-enriched molecule [78,79]. Readers can refer to ref. [82] for a pictorial description of hyperpolarization. It is performed in a ¹³C hyperpolarizer wherein microwaves transfer the polarization from electrons (trityl radical) at low temperature and high magnetic field to ¹³C-nuclei [83]. The hyperpolarized state lasts for only a couple of minutes, as the polarization decays back to its equilibrium level that is dependent on the spin-lattice relaxation time of the ¹³C-labeled atom. However, within this short time, the hyperpolarized molecule is injected into the subject, and the enhanced MRI signal can be observed within seconds. Several hyperpolarized ¹³C-labeled probes are now approved by the US Food and Drug Administration for use in the clinical imaging of humans. Hyperpolarized [1-¹³C] pyruvate was used to image the glycolytic metabolism in prostate cancer [84].

Hyperpolarized MRI was used in redox imaging using the hyperpolarized [1-¹³C] NAC probe [77]. The MRI using the hyperpolarized [1-¹³C] NAC probe revealed the global distribution of NAC, including the brain [77]. This is consistent with earlier reports using isotopically substituted NAC [10]. In both tumor cell and mice xenograft studies, [1-¹³C] NAC was shown to form a [1-¹³C] NAC–GSH dimer and other homodimers. This study also raised the possibility of an indirect mechanism by which NAC could induce GSH synthesis as opposed to the acylase 1-induced formation of cysteine and GSH, as there were no detectable levels of [1-¹³C] GSH [77]. The authors also suggest that the lack of detection of [1-¹³C] GSH could be due to a short relaxation time [85]. Other spectroscopic studies using stable isotopes of NAC did not provide any evidence for the presence of the corresponding ¹³C-labeled

GSH. NAC induction of GSH is cell dependent, and the mechanism of GSH formation remains to be fully established.

In summary, MRI using the hyperpolarized [1-¹³C] NAC probe may provide an understanding of the redox mechanisms of NAC in cancer therapy and in adaptive T cell immunotherapy, as discussed in subsequent section.

9. NAC-enhanced tumor metastasis

Therapeutic administration of antioxidants, which was once considered to be chemopreventive, now is thought to enhance tumor formation [86]. The role of ROS in cancer was suggested to be context dependent, as ROS, NRF2, and antioxidants promote or prevent tumor formation or progression [87].

Antioxidants may decrease oxidative damage in metastatic cancer cells, leading to their proliferation [88–90]. Due to its antioxidant mechanism, NAC may also accelerate the progression of some cancer growth.

The transcription factor BACH1, a master regulator of metastasis, is stabilized by NRF2 through induction of heme oxygenase [88]. NRF2 activation promotes lung cancer metastasis by inhibiting degradation of BACH1 [89]. NAC stimulates NRF2 under some conditions in tumorigenesis and tumor progression. Dietary antioxidants caused BACH1 stabilization and increased metastasis in a lung tumor model [91].

The effects of NAC often are discussed along with other antioxidants. Numerous agents, including mitochondria-targeted TEMPO (mito-TEMPO), vitamin E, and Trolox, inhibit metastasis cancer in preclinical models. As had been stressed in several review articles, ROS is not a single entity; rather, it represents many oxidants with totally different lifetimes and reactivities [6,92,93]. Thus, it is inconceivable that treatment with lipophilic antioxidants would exert the same effect as a hydrophilic antioxidant. Vitamin E is a chain-breaking hydrophobic antioxidant. Lipid peroxidation is a chain reaction where the chain-propagating radical is the lipid peroxy radical. Phenolic antioxidants like vitamin E scavenges the lipid peroxy radical that terminates the chain reaction and inhibits formation of lipid peroxides and lipid peroxide-derived aldehydes such as 4-hydroxynonenol and ferroptosis. Trolox is a water-soluble analog of vitamin E and reacts with oxidants like superoxide, hydroxyl radical, peroxy nitrite, nitrogen dioxide, and peroxy radical in the aqueous phase. The effects of Trolox should be distinctly different from those of vitamin E. MitoTEMPO is a nitroxide tethered to a positively charged triphenylphosphonium group through an alkyl side chain. The positively charged compound targets and localizes in mitochondria with a negative membrane potential [94]. Generally, its mechanism of action involves increased dismutation of superoxide to H₂O₂ [95], mitigating the reaction of superoxide with nitric oxide and forming peroxy nitrite, as well as the reaction of superoxide with iron sulfur proteins (aconitases). NAC exerts indirect antioxidant effects through mechanisms that are different than those of vitamin E, Trolox, and mitoTEMPO. Grouping all of these compounds under the umbrella term “antioxidants” causes confusion and hampers our understanding of their mechanisms of action [6,49,54].

Mitochondrial switch was suggested to promote tumor metastasis through generation of mitochondrial superoxide [96]. DCFH was used to monitor ROS in superinvasive tumor cells. Because of the inhibitory effects of NAC, mitochondria-derived ROS (superoxide and H₂O₂) were proposed to promote tumor cell migration and invasion. As discussed above, the DCFH-based fluorescence assay for H₂O₂ is fraught with problems; NAC inhibition of intracellular DCF fluorescence is not related to scavenging of ROS by NAC and is subject to multiple mechanisms affecting GSH and iron-dependent signaling pathways (Fig. 1).

10. Reaction between NAC and electrophiles in cancer cells

Dimethyl fumarate (DMF), the methyl ester of fumaric acid, is a US Food and Drug Administration-approved drug (trade name, Tecfidera)

for the treatment of relapsed forms of multiple sclerosis and psoriasis. The neuroprotective and immunomodulatory effects of DMF were attributed to the inhibition of the pro-inflammatory cytokines [97]. DMF is used as a pharmacological tool to enhance the anti-tumor effects of known chemotherapeutic agents [98,99]. DMF treatment induced necroptosis in colon cancer cells, and the proposed mechanism involved GSH depletion, enhanced ROS formation, and activation of MAPK-mediated signaling [100]. The effects of DMF were suggested to be more selective for cancer cells due to the increased GSH levels. NAC inhibition of ROS in cancer cells induced by DMF was attributed to enhanced GSH and the glutathione peroxidase-mediated antioxidant mechanism.

In other studies using pancreatic cancer cells, DMF inhibited cell growth and exacerbated metabolic cell death [101]. DMF-mediated effects were attributed to interruption of the tricarboxylic acid cycle and inhibition of mitochondrial respiration and glycolysis. NAC completely restored the mitochondrial respiration and abrogated the antitumor effects of DMF. DMF dose-dependently inhibited the folate metabolism enzymes that were restored by NAC. DCFH-DA (dichlorodihydrofluorescein diacetate) was used to monitor increased production of ROS in DMF-treated pancreatic cells. ROS-induced DCF fluorescence was found to be cell-specific in pancreatic cancer cells. At low concentrations of DMF, ROS-induced DCF fluorescence increased slightly, but it decreased at higher levels of DMF. Depletion of cysteine in pancreatic cancer cells induced ferroptosis [102]. Because NAC is known to inhibit ferroptosis, ROS-enhanced DCF fluorescence is likely to be related to ferroptosis. At low DMF concentrations, nuclear translocation of NRF2 and production of antioxidative enzymes increased, but the protective effects were lost at higher concentrations. DMF effects in cancer cells are apparently related to NRF2 [103]. The effects of NAC on DMF cytotoxicity in cancer cells may be indirectly related to changes in NRF2. Reversal of DMF effects in pancreatic cancer cells was not related to ROS. DMF-induced cell death in colorectal cancer cells involves hypoxia-inducible factor-2 α activation, enhanced iron signaling, and ferroptosis [104]. NRF2 is reported to be activated by the disruption of mitochondrial thiol homeostasis but not by enhanced mitochondrial superoxide production [105]. NRF2 activation causes metabolic rewiring and promotes tumor initiation and progression [106]. The paradoxical effects of NAC in cancer biology may be related to alterations in the KEAP1 (Kelch-like ECH-associated protein 1)/NRF2 pathway. The KEAP1/NRF2 pathway also controls glutamine metabolism and GSH synthesis in several tumor cells [17].

11. Immunomodulatory effects of NAC and anti-tumor immune function

CAR T cell therapy, a form of immunotherapy, involves using a patient's own T cells and genetically modifying them *ex vivo* such that they recognize a protein on cancer cells and kill the cells. The modified T cells, also known as CAR T cells, are returned to the patient to find and destroy the cancer cells. The CAR T cells often undergo enhanced oxidant-induced modifications that decrease their ability to multiply and attack cancer cells in the patient. These are referred to as exhausted T cells. Studies support the view that NAC treatment could reverse oxidant-induced changes and allow CAR T cells to multiply in the body [107–109]. NAC treatment significantly inhibited the DNA response pathway and the DNA damage marker. ROS was monitored by enhanced DCF fluorescence in T cell receptor stimulated cells that were diminished by NAC. These results are consistent with the involvement of peroxide and iron in T cell receptor stimulated cells, and inhibition by NAC could be attributed to a decreased iron and peroxide induced mechanism.

NAC improves the efficacy of adoptive T cell immunotherapy for melanoma. A study in a preclinical model of melanoma showed that culturing T cells in NAC before they are infused as immunotherapy improves the outcome [107]. NAC-cultured T cells were 33-fold higher than T cells cultured without NAC. NAC enhances the therapeutic

outcomes for adoptive cell transfer therapy by improving the anti-tumor function of exhausted T cells [107]. NAC enhances the antitumor functionality of T cells by activating PI3K/Akt and inhibiting Foxo1, and inhibiting ROS through indirect elevation of small hydrophobic proteins. GSH has been shown to prime T cell metabolism and T cell growth [110,111]. T cell proliferation is dependent on thiol-mediated regulation of interleukin-2 secretion. Reduced thiols, but not necessarily GSH, are required for T cell proliferation [112]. NAC has an opposing effect on T cells depending on the concentration. NAC promoted stem cell-like memory CD8⁺ T cells and exerted potent antitumor immunity [113]. The thioredoxin system plays a crucial role in modulating oxidative stress in regulatory T cells [114]. Enhanced surface thiols induced by the thioredoxin system were linked to its enhanced antioxidant function. A potential link between NAC and the thioredoxin system (Fig. 1) has also been investigated [115]. Modulating ROS levels was shown to regulate effector and memory CD8⁺ T cell differentiation, and NAC treatment inhibited the oxidation of CellROX and MitoSOX [113]. A role for mitochondrial superoxide was proposed based on the increase in MitoSOX-derived fluorescence in control cells and a decrease in MitoSOX fluorescence in NAC-treated cells. As emphasized in the recent review, enhanced fluorescence from ROS probes (HE and MitoSOX) should not be equated to enhanced superoxide (mitochondrial or cytosolic) without detailed analyses and determination of hydroxylated products specific for interaction with superoxide [6,49,93]. NAC inhibition of fluorescence is likely to be related to the other mechanisms discussed above. Recent research strongly implicates the ROS pathway controlled by thioredoxin 1 in T cell alloresponse and pathogenicity of graft-versus-host disease [116]. A Phase I study of NAC to optimize metabolic tumor microenvironment in CD19 CAR T cell therapy for lymphoma is ongoing (NCT05081479) [117].

12. Conclusions and future perspectives

NAC has been used as a nutritional supplement, and, currently, it is being used to treat chemotherapy (e.g., *cis-platinum*)-induced cognitive impairments. In this review, the mechanism of NAC as an ROS scavenging antioxidant has been critically evaluated, particularly in cancer biology and cancer immunology studies. NAC is contraindicated in people with bleeding disorders or who take blood thinners because it will slow blood clotting. A recently published consensus statement [6] articulates that ROS is not a single entity and that it must be properly identified using well-established and rigorously assessed methodologies. In nearly all of the experiments using NAC as an antioxidant, neither the identity of ROS nor its mechanisms have been determined. This review and the many preceding reviews [6,92,93] reveal the inadequacies and insufficient structural and mechanistic information of fluorescent probes and the oxidative products derived from them. A more comprehensive and rigorous understanding of the mechanism of action of NAC in cancer cells and ROS identification is needed, and should be determined using emerging and well-established assays [6]. Precision oncology and therapy require a rigorous understanding of the basic mechanisms and rigorous use of probes and analytical techniques. ROS (*i.e.*, superoxide and H₂O₂) play a dual role in cancer, and ROS-induced effects are cancer cell and cancer stage specific [24]. This implies that the effect of antioxidants, including NAC and NAC analogs, is context-dependent, which explains their paradoxical roles in preclinical cancer models. NRF2, a master regulator of antioxidant machinery, also exhibits a dual mechanism of action in different tumor stages [106].

N-acetylcysteine amide (NACA), a more membrane-permeable and blood–brain barrier permeant analog of the antioxidant thiol, NAC, was shown to be more potent than NAC in reversing paracetamol toxicity and oxidative stress-induced diseases [118–120]. NACA was shown to enhance the therapeutic effect of neural stem cell-based antiglioma oncolytic virotherapy [121]. The increased therapeutic efficacy of NACA was attributed to its ability to decrease ROS as measured by DCF fluorescence. The many interpretational concerns raised for NAC in the

current review also remain valid and applicable for interpreting results obtained using NACA.

Declaration of competing interest

None.

Data availability

No data was used for the research described in the article.

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