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Why Hydrogen Peroxide-Producing Proteins are Not Suitable Targets for Drug Development

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Abstract

Reactive oxygen species (ROS) play an important role in pathological processes and provide hope for the development of treatments aimed at suppressing the effects of hydrogen peroxide (H_2O_2). However, attempts to inhibit superoxide dismutase 1 (SOD1), the main antioxidant enzyme that converts superoxide anion into H_2O_2 and water during ROS metabolism, have not yielded significant results. To understand the reason for the failure, we studied the behavior of the epidermal growth factor receptor (EGFR) in cancer cells exposed to H_2O_2-generating compounds. EGFR can be activated by binding of EGF ligand to the extracellular region of the receptor and by interaction of H_2O_2-generating chemicals with the catalytic cysteine in the intracellular domain of the receptor. Both mechanisms independently trigger downstream signaling pathways in cells. EGFR expression can also be reduced by the protein tyrosine phosphatase PTP-1B, which itself is activated by H_2O_2. A simple in-gel fluorescence technique demonstrates the rapid binding of H_2O_2-generating molecules to hundreds of proteins in cancer cells. The natural defense system Nrf2 takes longer to break down target proteins and therefore cannot prevent H_2O_2 released by chemical agents from affecting unwanted proteins. It can be concluded that cytoplasmic SOD1 and other H_2O_2-producing proteins that protect cells from oxidative damage are not suitable targets for the development of practical drugs for the treatment of human diseases.

Keywords: Reactive oxygen species; hydrogen peroxide; superoxide dismutase; epidermal growth factor receptor; redox therapy; cancer

Introduction

Global ionizing radiation and photochemical reactions generate free radicals, suggesting that the evolution of oxygen-consuming organisms was driven by their ability to tolerate oxygen free radicals. A striking example of such evolution is the birth of a child, which represents the transition of the organism from a hypoxic maternal environment to the oxygen environment necessary for life. The action of free radicals requires a better understanding of the molecular mechanisms of tolerance, especially in the face of growing threats of climate change and military excesses.

The high redox potential of oxygen, which has two unpaired electrons in its outer orbit, allows it to serve as an ideal electron acceptor. The reduction of dioxygen (O_2) to superoxide radical ($O_2^{\bullet-}$) converts oxygen into free radicals known as reactive oxygen species (ROS), which attack various targets in the human body (Figure 1) [1]. The enzyme superoxide dismutase (SOD) was discovered as an important protein in the first line of cell defense against oxygen free radicals [2]. Among the three classes of SODs that have a distinct subcellular localization in eukaryotes, Cu/Zn SODs are expressed in the cytoplasm and extracellularly, and Mn SODs are expressed in mitochondria [3]. SOD directs the reaction of superoxide anion ($O_2^{\bullet}$) with the formation of hydrogen peroxide (H_2O_2) and oxygen ($O_{2)}$ according to the following reaction ($2O_2^{\bullet-} + 2H^+ \rightarrow H_2O_2 + O_2$) [4]. The resulting superoxide radical ($O_2^{\bullet-}$) is converted into a non-radical species with a high oxidation potential - hydrogen peroxide (H_2O_2), which can subsequently be transformed into a hydroxyl radical ($HO^{\bullet}$). Hydrogen peroxide decomposes into water and oxygen upon heating or in the presence of numerous substances, particularly salts of such metals as iron, copper, manganese, etc.

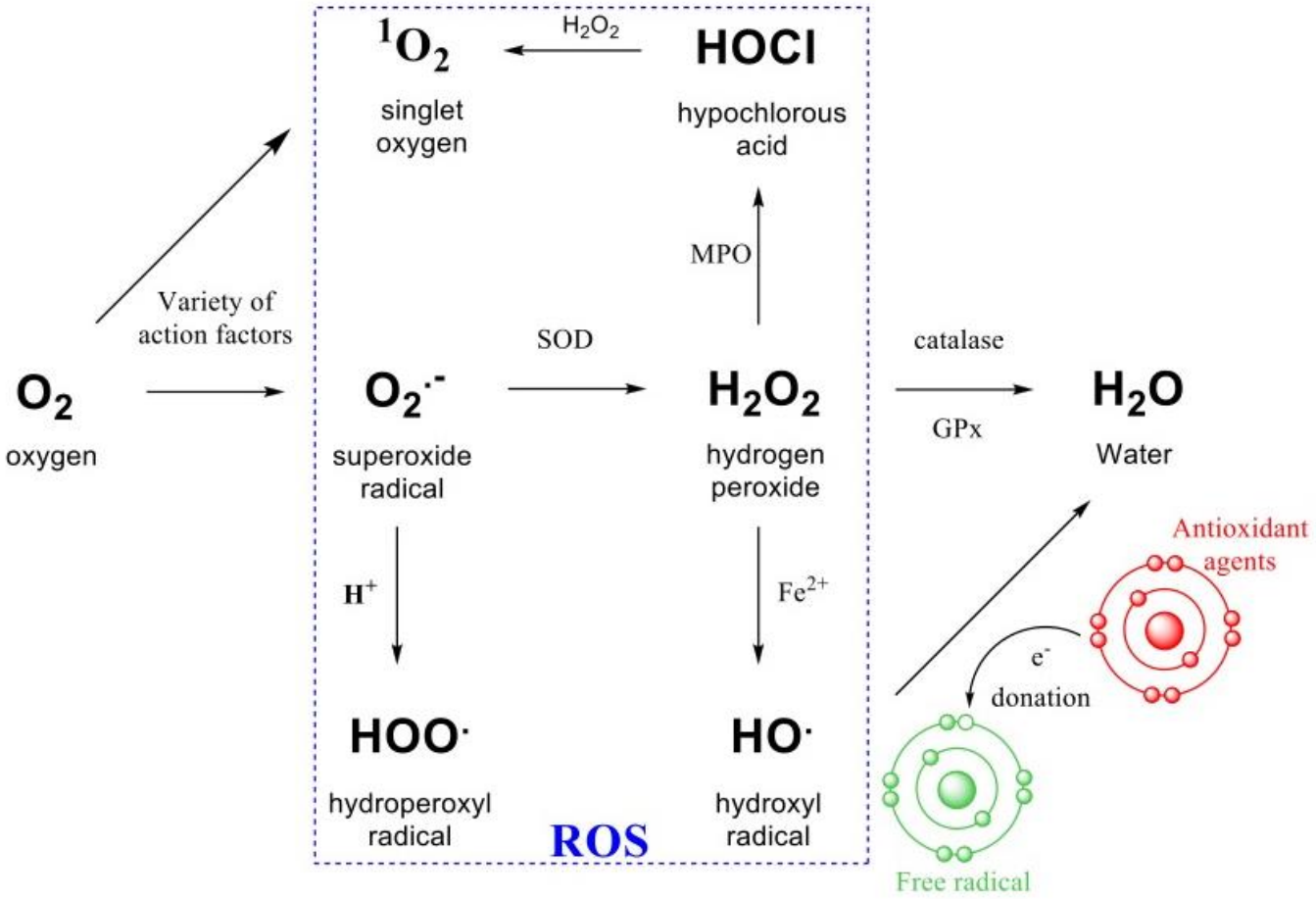


Figure 1: Intracellular conversion between different kinds of ROS and action of antioxidant agents. SOD - superoxide dismutase, MPO - myeloperoxidase, GPx - glutathione peroxidase, $Fe2^+$ is ferrous iron and participates in the Fenton reaction ($H_2O_2 + Fe^{2+} \rightarrow Fe^{3+} + OH\cdot + OH^-$). ROS radicals can generate a new, highly reactive and unstable radical on a cellular partner. The antioxidant stabilizes this radical by donating an electron to make it less reactive.

Three SOD isoforms are involved in many cellular functions, including the control of crosstalk in the cellular microenvironment, which has led to their extensive testing as possible therapeutic agents [5]. The presence of metal-containing cofactors in SOD isoforms allows the cell to maintain homeostasis and coordinate ROS signals between cellular compartments [6]. ROS generated in healthy cells can modify the functional building blocks of proteins and lipids, thereby preventing cell damage. However, in cancer cells, up- and down-regulation appears to be dysregulated, making ROS a hot topic in cancer research and anticancer drug development [7]. Both ligand-dependent and ligand-independent protein transactivations have been described, demonstrating that non-radical H_2O_2 plays a critical role in cellular functions, including signaling and metabolic pathways [8]. Oxidative stress theory suggests that ROS underlie disease states, which has prompted the proposal of in vitro and in vivo models to demonstrate their importance in the initiation and catalytic site inhibition of targeted proteins in human diseases [9].

The occurrence of cancer is often associated with disruption of the metabolic and regulatory functions of target proteins. Modern therapeutic treatment for cancer is carried out through a combination of treatments, including radiation therapy, surgery, immunotherapy and chemotherapy. Understanding the molecular mechanism of triple negative breast cancer, caused by the absence of three receptor proteins, namely estrogen receptor, progesterone receptor and human epidermal growth factor receptor 2 (HER2), has led to the consideration of chemotherapy as an effective treatment strategy. However, despite limited progress in the treatment of this and other malignancies, cancer therapy remains a global medical problem for humanity.

The fundamental role of H_2O_2 in the activation of proteins that direct signaling pathways in the cell indicates the need and possibility of developing therapeutic drugs against oncological, neurological and other diseases using the strategy of inhibiting target proteins. However, we question this traditional strategy for treating pathological processes in which the targeted H_2O_2-generating protein plays a key role in important host cell functions.

The Role of EGFR in Redox Processes

In humans, 58 proteins belong to the family of receptor tyrosine kinases (RTKs), involved in the phosphorylation of transmembrane proteins in cellular processes [10]. All RTKs are activated by a unique mechanism consisting of homodimerization and heterodimerization of the extracellular domain followed by autophosphorylation of tyrosine residues located at the C-terminus of the intracellular domain [11]. Phospho-tyrosine residues can be recognized by proteins bearing so-called SH_2 (SRC homology domain 2) and PTB (phospho-tyrosine binding domain) domains, which can implement the signal either by phosphorylation (e.g. JAK/STAT) or by recruiting docking proteins that initiate signaling cascades (e.g., RAS/MAPK and PI3K/AKT) in respective pathways as demonstrated for EGFR (Figure 2).

The EGFR gene, also known as HER1 (human epidermal growth factor receptor 1), encodes the EGFR protein, a transmembrane growth factor receptor that remains a popular target in drug development for cancer and other diseases [12]. The enormous amount of research carried out on the EGFR receptor since its discovery by Stanley Cohen, who together with Rita Levi-Montalcini received the Nobel Prize in 1986, has led to an understanding of the role of this protein in many life processes [13]. Numerous studies of this receptor have made it possible to understand the mechanism of its action and have opened up seemingly attractive prospects in the treatment of cancer.

Phosphorylation of proteins is regulated by the ratio of activities of the corresponding pair of protein kinase and phosphatase, which is modulated by hydrogen peroxide. Binding of the cognate ligand EGF to the extracellular domain of EGFR promotes dimerization of the receptor, which activates the ATP-binding catalytic site located in the cytoplasmic domain [14, 15]. This domain contains six Cys residues, and Cys797, located in close proximity to the ATP-binding pocket, is exposed to H_2O_2 [16]. The resulting H_2O_2 oxidizes the catalytic Cys797 to sulfenic acid in EGFR, which enhances autophosphorylation of the receptor [17]. Unstable sulfenic acid is further oxidized to stable sulfinic acid (Cys-SO_2H) without loss of receptor kinase activity [18, 19]. Protein tyrosine phosphatase (PTP), activated by H_2O_2, also can inhibit EGFR tyrosine phosphorylation [20]. PTP-1B is the main dephosphorylating enzyme of target proteins, and the action of H2O2 leads to the nucleophilic oxidation of cysteine to the unstable elec-

trophilic sulfenic acid (Cys-SOH), which causes the formation of inactive 3-isothiazolidinone at the catalytic center [21-23]. Consequently, the degree of oxidation of the catalytic cysteine in EGFR itself and in various PTPs determines the efficiency of downstream signaling pathways in cells [18, 24].

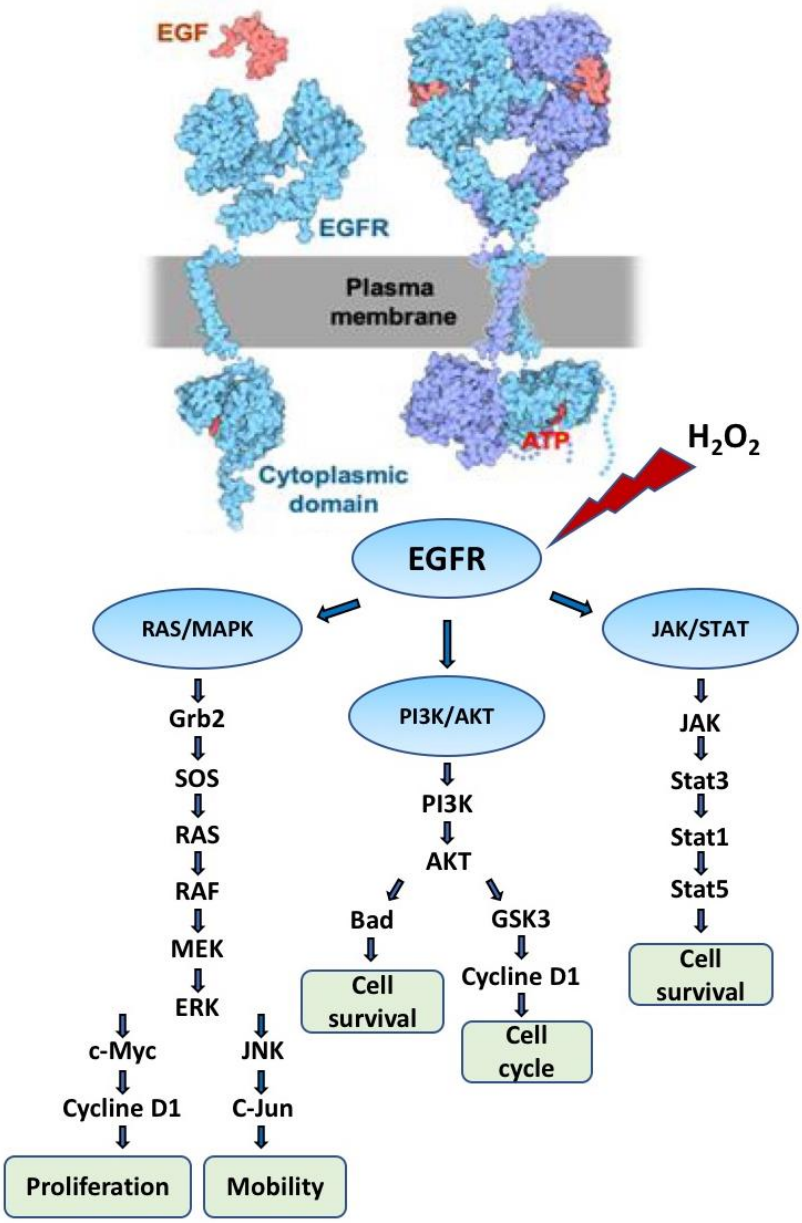


Figure 2: Control of signaling pathways by EGFR. Extracellular binding of the cognate ligand EGF to EGFR results in dimerization of the receptor, followed by activation of the ATP binding site, phosphorylation of receptor tyrosine residues, and initiation of signaling pathways (three major pathways shown). H_2O_2 phosphorylates EGFR in the absence of EGF ligand or in competition with EGF in the culture medium, resulting in activation of downstream signaling pathways. Activated PTP-1B and possibly other phosphatases also contribute to EGFR inhibition.

To determine whether EGFR activation depends on the dimerization state of SOD1, the level of receptor tyrosine phosphorylation was assessed using an siRNA interference experiment [25]. The amount of both monomeric and dimeric forms of SOD1 was significantly reduced in cells after 5 min of exposure to H_2O_2-producing compounds compared to non-transfected cells or cells treated with scrambled siRNA. Therefore, SOD1 activity is critical for EGFR-mediated tyrosine phosphorylation. H_2O_2 also activates EGFR signaling in the absence of EGF ligand in the growth medium, which leads to an increase in the amount of tyrosine phosphorylated proteins pShc, pERK1/2, pGab1 and pCBL in downstream signaling pathways [25].

The fluorescent electrophilic molecule 4-nitro-2,1,3-benzoxadiazole (NBD), also called nitrobenzofurazan, has an excitation wavelength (λex) of 440 nm and an emission wavelength (λem) of 480 nm and has been used as a source of hydrogen peroxide for the evaluation of functional groups of proteins, development of molecular probes and fluorescent dyes for cell imaging [26-28]. As shown in Figure 3A, a compound containing the NBD scaffold NSC 228155 induced phosphorylation of eight RTKs treated for 10 min, namely EGFR1, ErbB2, ErbB3, insulin R, IGF-IR, Mer, ROR1 and EphA1, by the method protein microarrays on a nitrocellulose membrane [25]. Compared with the level of EGFR phosphorylation induced by H_2O_2, the EGF ligand also exhibited the highest binding ability to this receptor and weaker interaction with other RTKs (Figure 3B). This suggests that both the ligand protein and the NBD chemical have a fairly similar ability to phosphorylate and activate the same spectrum of RTKs through two different mechanisms, binding the cognate EGF ligand to the extracellular domain of the receptors and influencing their intracellular domain via H_2O_2.

Thus, two mechanisms trigger RTK-dependent signaling pathways that are important for cell adaptation to different environmental conditions. As previously established for EGFR, other RTKs can be also activated by binding a ligand protein that dimerizes the extracellular region of the receptor and by targeting an H_2O_2-generating chemical molecule to the catalytic Cys797 residue in the intracellular region of the protein.

The sequential action of three enzymes, namely superoxide dismutase, catalase and glutathione peroxidase, protects cells from the action of toxic forms of ROS. Considering that the activity of these enzymes occurs in dimeric and possibly higher oligomeric forms, we assessed the oligomeric state of the corresponding enzymes in breast and prostate cancer cells treated with ROS [29]. It turned out that a significant part of the 16 kDa Cu/Zn-SOD1 monomer was combined into a 32 kDa dimeric structure in cells exposed to ROS for 5 min. At the same time, no changes in the molecular weight of catalase and glutathione peroxidase were detected in cells.

Lipophilic derivatives of NBD rapidly bind to SOD1, forming a stable dimeric protein in the absence of adequate catalase activity in cancer cells [29]. In the absence of a subsequent coupled redox reaction, non-reactive H_2O_2 should theoretically accumulate in the cytoplasm under the influence of SOD1. But H_2O_2 can be converted into a reactive form of ROS, namely the hydroxyl radical $HO^{\bullet}$, if the extracellular conditions change and catalase or another adequate oxidative reaction ensures this conversion.

The role of SOD1 in cell signaling was also proven for the DNA protein kinase catalytic subunit (DNA-PK) in prostate cancer cells exposed shortly to the compound NSC 228155 [30]. The DNA-PK is a key protein involved in the repair of DNA double-strand breaks, which are considered the most cytotoxic DNA lesions and result from endogenous events such as the production of ROS during cellular metabolism [31]. It was found that tyrosine phosphorylation as well as the expression and activity of DNA-PKcs drastically decreased in cells exposed to NBD compounds for 10 min. Of note is that this decrease was accompanied by increased protein ubiquitination and the activation of the proteasome machinery leading to the protein degradation [30].

For nearly three decades, EGFR has served as an attractive target for drug development against various types of cancer through the strategy of inhibiting protein activity [12]. Reduction of EGFR activity by chemically synthesized inhibitors is usually due to the effect on Cys797, which leads to improved well-being of patients. However, the effect of EGFR inhibitors is characterized by a short--term therapeutic effect due to the occurrence of mutations in patients, which in clinical studies become resistant even to third--generation drugs with nanomolar activity [32]. Thus, the bottleneck in inhibitor therapy is the emergence of resistance mutations in EGFR or other parts of the tumor tissue, which precludes further cancer chemotherapy.

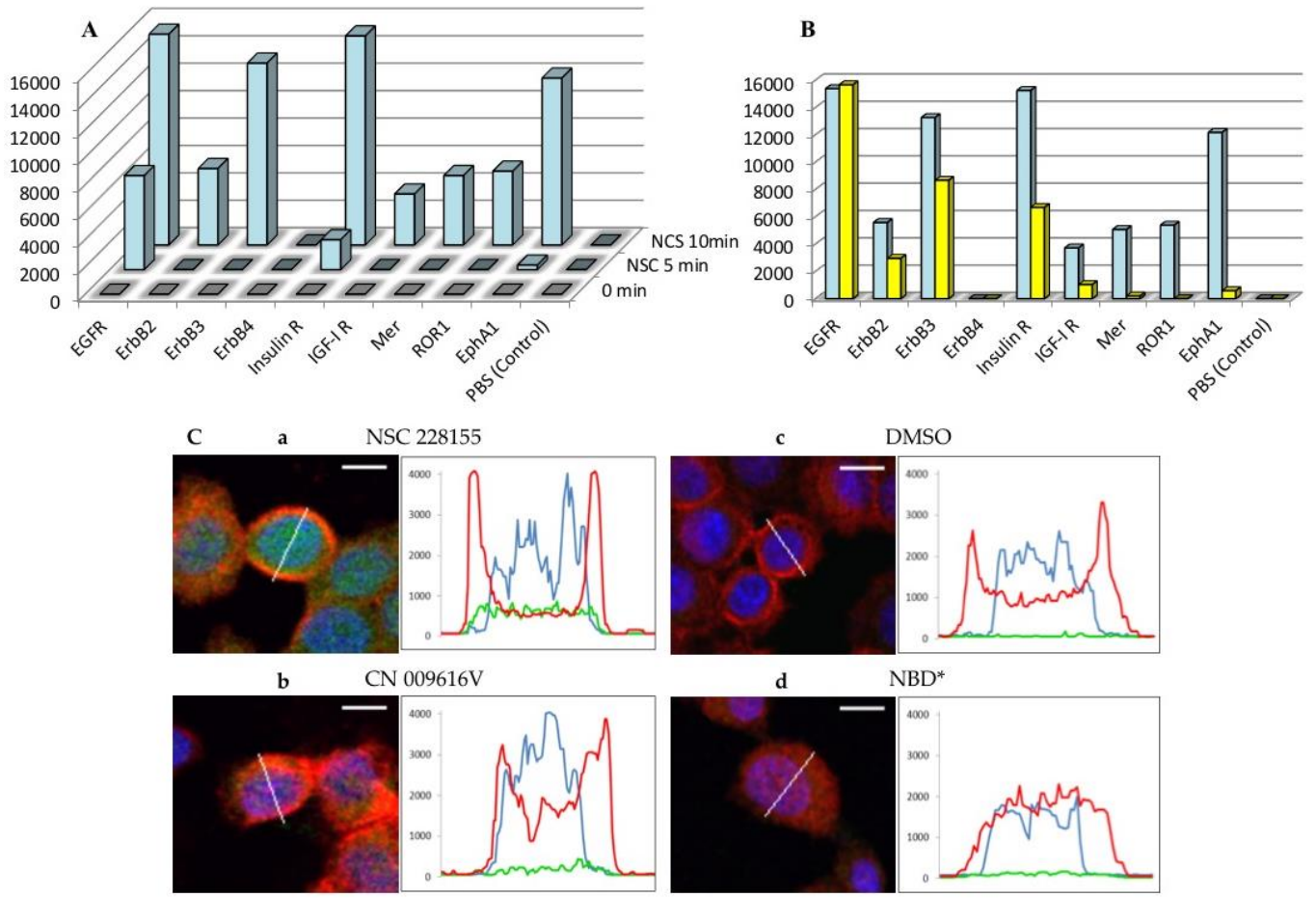


Figure 3: Time-dependent RTK activation in breast cancer cells exposed to NSC 228155 (A) and compared with EGF (B) [25]; areas of cells affected by the NBD compound as detected by fluorescence microscopy (C) [29]. Fluorescence images of cells (left in C) and graphical distribution of fluorescence in cells (right in C) exposed to NBD compounds. The wavy line shows the area being scanned; bar -10μ. Fluorescence of NBD compounds (emission 520 nm) is shown in green, plasma membrane (650 nm) in red, nucleus (420 nm) in blue.

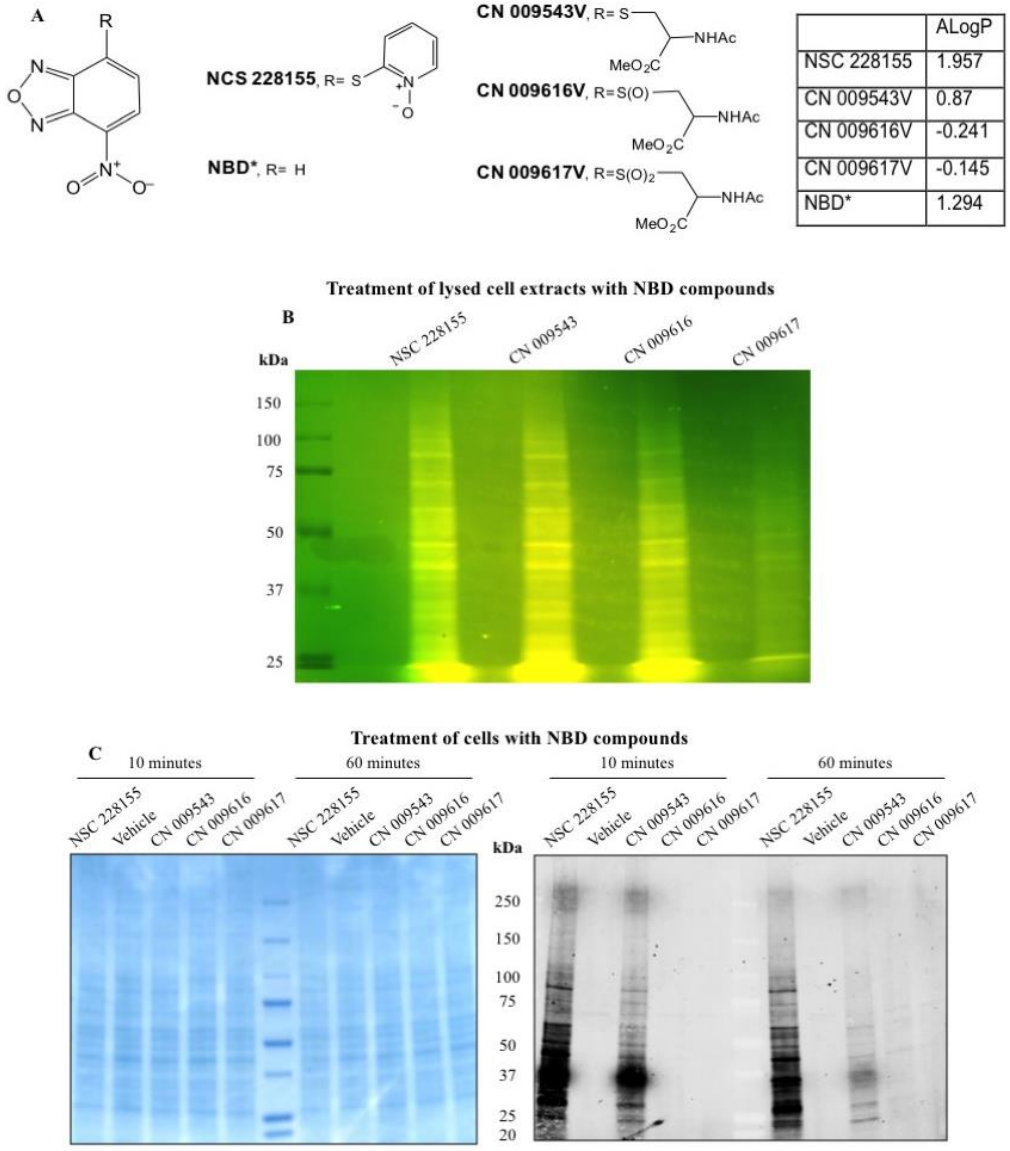
Straigthforward Detection of Hydrogen Peroxide Binding to Many Proteins

We took advantage of the fluorescence of the NBD structure to determine how its derivatives enter cells. Signals of different colors for the compound, plasma, and nucleus were detected using fluorescence microscopy and recorded as curves of different colors. NBD compounds have different abilities to penetrate cells [29]. Compound NSC 228155 penetrates cell membranes and disperses in both the cytoplasmic and nuclear compartments, whereas compound CN 009616V cannot penetrate the lipid bilayer (Figure 3C). A compound containing only the NBD scaffold severely damages the plasma membrane at the concentration used. This behavior is consistent with the lipophilicity of NBD compounds according to the calculated logP value (Figure 4A). The compound having a positive logP can enter cells and enhance tyrosine phosphorylation of EGFR in cells. In contrast, a compound with negative logP cannot cross the plasma membrane and therefore does not induce phosphorylation. The differential effects of NBD compounds on the cell can be important for subsequent understanding of the effect of hydrogen peroxide on the phosphorylation of many proteins.

A wide range of modern methods are used to detect ROS and their actions, including fluorescent [33] and chemiluminescent assays [34], chromatographic [35] and spectrophotometric assays [36, 37], electrochemical biosensors [38] and electron paramagnetic resonance [39]. Small fluorescent molecules capable of generating H_2O_2 have proven important for the simultaneous detection of a large number of interacting partners in living cells.

We turned to the principle of immobilization of proteins on a nitrocellulose membrane with subsequent recognition of the reacting proteins by fluorescent antibodies [40,41]. However, in the method, known as "in-gel" [42], target proteins can be detected using fluorescent compounds that bind to proteins directly in the cell, without the need for a specific antibody binding step.

A fluorescent signal was detected in all four protein samples tested in lysed extracts of MDA MB-468 breast cancer cells when all proteins were accessible to NBD compounds (Figure 4B). In contrast, a fluorescent signal was detected after 10 min and 60 min exposure of native cells to only the lipophilic compounds NSC 228155 and CN 009543 (Figure 4C) [43]. The observed decrease in fluorescence intensity of bound proteins after 60 min of treatment (see Fig. 4C) may be due to degradation of the reacting proteins or dye inactivation. It is appropriate to note that both lipophilic compounds NSC 228155 and CN 009543, having different structures, bind to many proteins of a similar spectrum with a molecular weight from 10 kDa to almost 400 kDa.



	ALogP
NSC 228155	1.957
CN 009543V	0.87
CN 009616V	-0.241
CN 009617V	-0.145
NBD*	1.294

Figure 4: Structure and ALogP values of NBD compounds (A) [29] and binding of NBD compounds to proteins in lysed cell extracts (B) and native cell extracts detected by in-gel method (C) [43]. An image of proteins stained with Coomassie blue (left in C) and an image of many proteins detected on a nitrocellulose membrane in a fluorescence scan at λex 488 nm and λem 520 nm (right in C). The membrane was scanned using a Typhoon 9410 imaging system (Molecular Devices) equipped with filters of different wavelengths.

This suggests that binding to proteins is carried out by hydrogen peroxide common in the structure of the compounds, and not by another part of these compounds. In addition, the intensity of the fluorescent signal indicates that there are likely to be 15 highly expressed target proteins in breast cancer cells, and the total number of moderately and weakly expressed targets attacked by H_2O_2 is likely to exceed hundreds [44]. In this regard, mass spectrometry studies of the human redoxome have identified 900 sites of sulphenylated cysteine residues, found primarily in proteins that play antioxidant or other metabolic roles in the cell [45].

H_2O_2regulates the expression of transcription factors through both activation and inactivation in cells [46]. The identification of H_2O_2-exposed proteins by in-gel technique requires further detailed study. In addition to the known proteins, including SOD, tyrosine phosphatases and H_2O_2-producing/generating proteins, the expected list will contain others that humans need to survive in an oxygen-rich atmosphere. These unknown proteins are of fundamental interest for understanding human evolution and discovering reliable targets for the development of truly effective therapeutics.

Nrf2-ARE redox pathway

The cellular system for regulating ROS levels was discovered by isolating the transcription factor Nrf2 (nuclear factor E2-related factor 2), which allows the assessment of increased amounts of free radicals [47]. Constitutive activation of Nrf2 plays an important role in oxidative stress resistance, metabolic reprogramming, ferroptosis inhibition, and chemotherapy resistance of cancer cells, and is associated with poor prognosis and survival [48, 49]. Nrf2 consists of seven Nrf2-ECH homology (Neh) cap “n” collar (CNC) domains (Fig. 5). Recognition and binding of Nrf2 to sensitive genes is determined by the antioxidant response element EpRE/ARE, the activity of which is determined by the state of the redox-sensitive inhibitor Keap1 (Kelch-like ECH-associated protein 1), which acts as an adapter for the cullin E3 ubiquitin ligase [50]. With increasing oxidative stress, Keap1-reactive cysteine residues are oxidized, resulting in conformational changes that inhibit Nrf2 binding to Keap1 and allow the protein to escape proteolytic degradation.

With increasing oxidative stress, Keap1-reactive cysteine residues are oxidized, resulting in conformational changes that inhibit Nrf2 binding to KEAP1 and further movement of Nrf2 toward ubiquitination. Nrf2 determines the antioxidant response by binding to the ARE, a cis-acting regulatory element responsible for the expression of detoxification enzymes [51]. In the absence of oxidative stress, Nrf2 is ubiquitinated and degraded with a half-life of 30-60 min by the ubiquitin-proteasome system. Under conditions of oxidative stress caused by reactive electrophiles, toxins, or ARE inducers, the interaction between Nrf2 and Keap1 is disrupted and Nrf2 translocates into the nucleus, where it binds to small Maf proteins, increasing the rate of transcription of recognized genes. Thus, electrophilic compounds producing hydrogen permease covalently modify cysteine residues in the Keap1 protein and activate Nrf2, which triggers an antioxidant response and protects cells from oxidative stress.

A recent article describes the variety of beneficial effects of electrophilic compounds that interact with target proteins [52]. Nrf2 plays a critical role in maintaining cellular redox homeostasis and regulating cellular antioxidants such as glutathione and thioredoxin, as well as stimulating the expression of enzymes involved in NADPH and ROS regeneration, xenobiotic reduction and detoxification [53, 54]. Nrf2 is the main regulator of cytoprotective processes, which can lead to the development and progression of human diseases, including cancer. Its expression closely correlates with drug resistance of tumour cells [55]. In colorectal cancer cells with Nrf2 knockdown, changes occurring in signalling pathways were associated with loss of ROS scavenging and detoxification potential [56]. In the absence of Nrf2, certain activated pathways such as MAPK, JNK and FOXO appear to reduce the deleterious effects of redox deficiency, and as knockdown cells become more sensitive to drugs, these pathways may be targeted to treat cancer. These observations do not exclude the possibility of activation of pathways other than Nrf2-ARE, which may contribute to a cytoprotective response against ROS and electrophiles [57].

Typically, the Nrf2 regulatory system takes about an hour to transcribe the Nrf2/Keap genes and translate the corresponding protein that recognizes the oxidant H_2O_2. The in-gel method quickly, in almost five minutes, detects binding to a large number of proteins that react differently to hydrogen peroxide. This means that the natural Nrf2 defence system, which takes longer to activate,

appears to be unable to prevent H_2O_2 from binding to proteins and protect the human body from exposure to unwanted proteins. In this context, the decrease in fluorescence intensity of some NBD-interacting proteins after 60 min of treatment (see Fig. 4C) appears to be the result of their delayed degradation by the Nrf2 system.

Other stress-responsive transcription factors are induced by the same types of reactive toxicants as Nrf2, indicating that the overall cellular response to oxidants also involves other pathways such as HSF1 and HIF1 [58]. Cellular stress perception and defence activity are regulated by these three oxidant-activated transcription factors HSF1, HIF1 and Nrf2, which control the heat shock response (unfolded proteins), hypoxia response and antioxidant response, respectively [59,60]. These three system responses create a multi-layered cellular defence consisting primarily of non-overlapping programs that mitigate the limitations of each response.

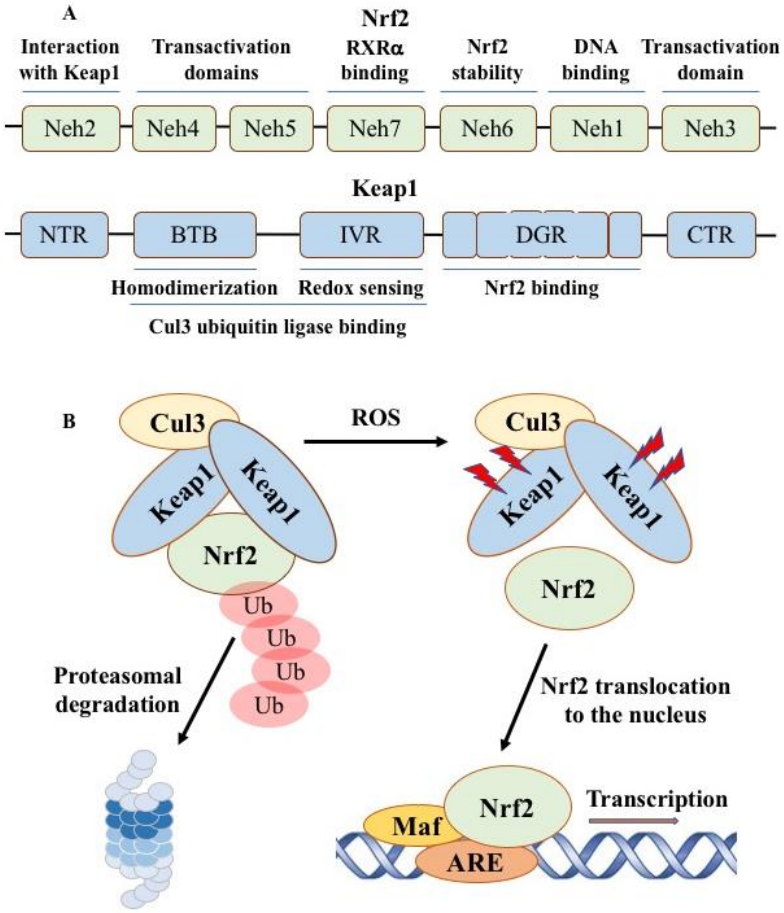


Figure 5: Domain structure of Nrf2 and Keap1 proteins and Nrf2 activation in response to ROS stress conditions. In the absence of stress, the Nrf2 protein complex is ubiquitinated by the Cul3 ubiquitin ligase and Nrf2 continuously degraded by the cellular proteasome. In response to ROS stress, Keap1 is inactivated, which leads to stabilization of Nrf2 and its passage into the nucleus and dimerization with small Maf proteins, followed by binding to the antioxidant response element ARE to activate transcription of various target proteins.

Therapeutic Opportunities by Targeting ROS-Producing Proteins

SOD protein is involved in various cellular functions such as proliferation, survival, invasion, angiogenesis, migration, apoptosis and death. The response to ROS-induced events is complex, and disruption of cellular regulation can cause disease in humans [61, 62]. Therefore, SOD has attracted medical interest as a convenient target for chemical compounds that could become drugs against cancer, neurological diseases, skin allergies and other pathological processes. However, whether H_2O_2 production can be inhibited to prevent disease remains an important but controversial issue.

Human cells use a scavenging system in the cytoplasm, mitochondria and extracellular matrix, including superoxide dismutase, glutathione peroxidase, glutathione reductase, peroxiredoxin, thioredoxin and catalase, as well as other antioxidants that convert superoxide anions into water [63]. Endogenous ROS generated during inflammation can cause chemical damage to macromolecules, including DNA, proteins and lipids. Damage to intracellular macromolecules leads to oxidative stress during cellular respiration or inflammation under aerobic conditions. In the DNA structure, the guanine base is the most electron-rich and the most prone to oxidation, which is converted into several oxidation products, the main one being 8-oxo-7,8-dihydroguanine. When DNA is damaged by free radicals, the amount of 8-oxo-7,8-dihydroguanine increases, which has made it possible to use this molecule as a biomarker of oxidative stress in various diseases [64]. Oxidative damage to proteins affects their activity, leading to increased targeted proteolysis and dysfunction of signaling pathways in cancer [44, 65]. As a result of lipid peroxidation by ROS, the resulting lipid radicals, alkanes and isopropanes affect the course of the pathological process, especially in diabetes and neurodegenerative diseases [66].

Biological thiols such as glutathione and N-acetylcysteine also play an important role in maintaining redox homeostasis by scavenging free radicals [67]. Glutathione is a tripeptide antioxidant composed of glutamic acid, cysteine and glycine and is part of the intracellular defense against ROS. N-acetylcysteine maintains the level of -SH group in the cysteine structure necessary for glutathione. In redox homeostasis in healthy cells and tissues, the level of oxidized disulfide glutathione is maintained at low levels, while reduced thiol glutathione remains high. Therefore, an increased glutamine disulfide/thiol ratio can be considered an indicator of oxidative stress [68, 69].

Antioxidant enzymes are H_2O_2 sensors that regulate the progression of inflammation in pathological disorders [70, 71]. Natural foods are considered potential antioxidants if they counteract the effects of oxidative stress on disease pathogenesis and aging. For example, bee products such as honey, propolis and royal jelly act as radical scavengers and are considered natural antioxidants [72, 73]. Therefore, evaluation of the antioxidant activity of products is necessary to select the best samples that meet the requirements of medical use.

It is pertinent to note that synthetic antioxidants are widely used as additives to plastics, rubbers, cables and other polymeric materials to prevent oxidative degradation of these products [74, 75, 76]. This means that large-scale production of synthetic antioxidants results in the release of ROS into the atmosphere, which can trigger ozone layer depletion and subsequent undesirable effects on life on this planet [77].

Hydrogen peroxide in low concentrations is used as an antiseptic for minor cuts. The US Food and Drug Administration (FDA) classifies hydrogen peroxide as GRAS - generally recognized as safe - for humans at low doses. However, H_2O_2causes irritation, burning and blistering of the skin. The FDA does not recognize it as a dietary supplement. In February 2022, Medical Faculty Associates reported that the claim that topical application of hydrogen peroxide to the skin can treat cancer is unfounded [https://gwdocs.com/news/fact-check-false-claim-rubbing-hydrogen-peroxide-skin-treats-cancer-usatodaycom].

Smoking continues to be the leading risk factor for cancer worldwide, as tobacco smoke contains thousands of chemicals, including more than 70 carcinogens [78]. Smoking causes chronic and progressive lung inflammation, which is a key factor in the pathophysiological progression of cancer [79, 80]. The US federal government estimated that in 2023, nearly 2 million Americans would be diagnosed with cancer (other than nonmelanoma skin cancer) and more than 600,000 people would die from the disease [https://www.aacr.org/patients-caregivers/awareness-months/national-cancer-prevention]. More than 40% of these cases and almost half of the deaths can be attributed to preventable causes - smoking, excessive sun exposure, excess body weight and lack of physical activity. That is, the potential role of ROS, including hydrogen peroxide, may be significant in the actual cause of cancer. Suggested measures such as quitting smoking and protecting your skin from the sun may help reduce the risk of cancer caused by ROS.

Our data suggest that H_2O_2, through interaction with the catalytic cysteine of EGFR, acts as a physiological mediator of signaling

in cancer cells [25, 29, 44]. Acquired clinical resistance to EGFR inhibitors in cancer usually results from a tertiary mutation combining the C797S mutation, MET amplification and KRAS mutations [81], of which the EGFR C797S mutation is the dominant one [82]. Attempts to eliminate the receptor upon expression of the C797S mutation in triple mutant EGFR cells using allosteric degraders were unsuccessful [83].

A number of laboratories were forced to stop research on the creation of new antioxidant drugs against a number of diseases due to the lack of a positive effect on proteins of therapeutic importance, such as glutathione S-transferase, c-Myc oncogene and HIV integrase [84, 85, 86]. The powerful antioxidant edaravone was originally developed as a neuroprotective agent for acute cerebral infarction and neurological pathologies [87]. But after trials, it became attractive only for the treatment of amyotrophic lateral sclerosis. Data from animal models indicate its possible safety in humans [88]. However, the lack of clinical trial data does not allow us to talk about the real therapeutic value of edaravone.

Thus, reliable drugs that stop the progression of oncological, neurological, allergic and other disease models due to the influence of H_2O_2-generating compounds have not been described. The failure to effectively modulate H_2O_2 activity with chemical compounds in laboratory and clinical trials suggests that the problem is more complex than previously thought. It can be assumed that the stumbling block to successful therapy is not so much the acquisition of resistant mutations in the gene encoding SOD1 or another H_2O_2 producing gene, but the ineffectiveness of the protein inhibition strategy itself.

On what basis do we come to this conclusion? Considering the presented data, the following observations can be noted. First, the compounds that generate H_2O_2 bind to a huge number of proteins that perform many different functions. Secondly, among the target proteins there are those whose significance may change due to their biological nature, acquired mutations or other intracellular reasons. Third, activation of the Nrf2 defense system in cells requires a longer binding time to unwanted proteins than the shorter binding time of H_2O_2-generating chemicals to target proteins. Fourth, H_2O_2 can play dual and opposing roles in its action, activating some proteins and inactivating others that are critical for many cellular functions. Fifth, it cannot be ruled out that the effect on the disease process may change during treatment when the activity of target proteins is modulated by changes in growth conditions. Sixth, H_2O_2 acts on a cysteine residue located in close proximity to the catalytic site of EGFR and possibly other RTKs, resulting in the simultaneous performance of different important functions by the same chemical agent. Thus, the current lack of reliable drugs to counteract the undesirable effects of reactive ROS confirms the unsuitability of the strategy of inhibiting proteins producing H_2O_2 for the treatment of cancer and other diseases.

What molecular biology and synthetic chemistry strategy could provide pharmacological treatment for patients with diseases aggravated by unbalanced ROS action? We believe that when treating cancer with chemical agents, one should not strive to maximally inhibit the catalytic site of EGFR, thereby stopping various functions necessary for the life of the cell. This situation can be overcome by reducing EGFR activity; for example, by blocking the allosteric site in close proximity to the catalytic site of EGFR, which leads to inhibition of the downstream Bim-promoted pathway and cytoskeletal dysfunction, followed by cell detachment from the extracellular matrix and ultimately cancer cell death [89, 32]. That is, limiting the intracellular H_2O_2 content will allow the cancer cell to at least perform the functions necessary for survival. However, this assumption requires further confirmation.

Another cancer treatment approach uses a new strategy known as targeted protein degradation (TPD) to remove proteins, including EGFR, from diseased cells [90-92]. Several TPD-based protocols have entered early stages of clinical trials [93]. However, we believe that TPD is not suitable the degradation of SOD1 and other H_2O_2 metabolizing proteins in a clinical setting for the same reasons described above using a targeted protein inhibition strategy.

Another innovative strategy is based on immunological blockade of the functions of the intracellular region of transmembrane proteins using monoclonal antibodies to the extracellular region [94, 95, 96]. Chimeric antigen receptor (CAR) T-cell therapy has emerged as a promising alternative to catalytic site inhibition in cancers caused by mutant EGFR [97, 98]. However, CAR-T cell therapy causes serious side effects due to on-target/non-tumor toxicity, resulting in limited clinical efficacy [99,100]. This obstacle

has recently been overcome by a bioengineering strategy to create an active form of anti-EGFR CAR that specifically targets the activated receptor only in tumor cells and not in healthy tissues [101]. It should be speculated that the therapeutic effect provided by inhibition of the extracellular region of EGFR is an immunologically attractive strategy for treating disease if the catalytic site is not or weakly affected. This can only be assessed after clinical trials.

Thus, scientific evidence and analysis of available clinical studies indicate that chemotherapy cannot actually stop cancer progression in patients by blocking superoxide dismutase, which produces H_2O_2, and the receptors whose catalytic site is activated by H_2O_2. Currently, combination therapy appears to be a more reliable solution to the problem than simple chemical elimination of the activity of proteins that produce or utilize hydrogen peroxide, which is necessary for the normal functioning of cells.

Conclusions

H_2O_2 plays a key role in redox signaling pathways, promoting homeostatic metabolism or toxic reactions in cells. Therefore, understanding the effect of a potential therapeutic agent on the target protein(s) is an important task when choosing a strategy for its action against a specific disease. Free radicals formed by the interaction of an electron with active oxygen contributed to the emergence of intracellular H_2O_2 and the subsequent evolution of aerobic life. In the absence of progress in the development of real drugs that counteract the negative effects of reactive ROS, the question arises about the need to create chemical and biotechnological structures that inhibit superoxide dismutase.

The published data allows us to emphasize that the criterion of maximum inhibition of EGFR activity, popular among researchers, is not appropriate in medicine when assessing the effect of chemicals on the catalytic center of the receptor. Complete inhibition of EGFR in cancer cells means stopping many of the receptor's important functions in diseased and healthy cells. Therefore, the duration of the beneficial effect is of greater importance as a realistic criterion for proposed drugs in the treatment of cancer caused by overexpressed mutant or dysregulated EGFR, in which the catalytic site is activated by H_2O_2.

In summary, the important role of SOD in many cellular processes is inconsistent with the inhibitory effect of a potential chemical drug against cancer and other diseases if it completely eliminates the function of hydrogen peroxide in cells.

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Conflicts of Interest

The authors declare no conflict of interest

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