Role of SOD Peroxidase Activity in Oxidizing Drugs: Potential Modulation of Drug Toxicity

by

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Abstract

There is a close relationship between inflammation and cancer. At site of inflammation, reactive oxygen species (ROS) are generated, including hydrogen peroxide (H_2O_2). The generation of ROS could, in turn, activate transcription factors that are involved in inflammation, cellular transformation, tumor cell death, proliferation, angiogenesis, and metastasis. However, redox-buffering systems work as a cellular defense against ROS. Exhausting ROS-buffering capacity generates oxidative stress that are involved in many diseases, including cancer. The activities of two major antioxidant enzymes, superoxide dismutase (SOD) and catalase (CAT), are decreased in cancer tissues. The potential accumulation of H_2O_2 is known to inactivate SOD but with the presence of certain intermediates, such as bicarbonate (HCO_3^-), the induction of oxidative stress can be propagated through carbonate radicals ($CO_3^{\bullet-}$); this is called SOD peroxidase activity. It is not known, however, if SOD peroxidase-derived $CO_3^{\bullet-}$ can oxidize the drugs and consequently modulate their metabolism and cytotoxicity.

We investigated the oxidation of phenylbutazone (PBZ) and 6-mercaptopurine (6-MP) by SOD peroxidase-derived $CO_3^{\bullet-}$ through utilizing biochemical assays including, electron paramagnetic resonance (EPR)-spin trapping, oxygen analysis, UV-Vis and LC/MS measurements. Monitoring SOD protein and its activity and demonstrating the cytotoxic effect of oxidizing PBZ and 6-MP on cancer cells was also investigated.

We found that SOD peroxidase activity was significantly attenuated by increased

concentrations of PBZ that culminated in PBZ carbon-centered radicals as judged by computer simulation of hyperfine splitting constants. An identical PBZ carbon-centered radical was detected by reaction of PBZ with other peroxidases such as myeloperoxidase (MPO/H_2O_2) and horseradish peroxidase (HRP/H_2O_2). The oxygen uptake was significantly increased when PBZ was oxidized by SOD peroxidase activity, further confirming PBZ carbon radical formation. Both 4-hydroperoxyphenylbutazone (4-hydroxy-PBZ) and 4-hydroxyphenylbutazone (4-OH-PBZ) were detected after oxidizing PBZ by SOD peroxidase activity as evidenced by both UV-Vis and LC/MS analysis.

By doing further investigations, we found that diethyldithiocarbamate (DDC), SOD inhibitor, inhibited SOD peroxidase activity. Also, the PBZ carbon-centered radical was significantly attenuated by glutathione (GSH) that resulted in thiyl radical formation. Sorbic acid decreased PBZ carbon-centered radicals in concentration-dependent manner. GSH, but not sorbic acid, protected SOD protein from oxidation by $CO_3^{\bullet-}$ radicals. The cytotoxicity of PBZ was synergistically enhanced with the presence of H_2O_2 compared with either H_2O_2 or PBZ used alone. However, the synergistic cytotoxic effect of combined treatment on HepG2 cells was significantly attenuated by the presence of DDC. And extracellular addition of both human and bovine SOD significantly catalyzed the cytotoxic effect of combined PBZ with H_2O_2 on HepG2 cells.

We also found that SOD peroxidase-derived $CO_3^{\bullet-}$ radicals catalyzed the oxidation of 6-MP by bicarbonate-activated peroxidase system (H_2O_2/HCO_3^-). The oxidation of 6-MP by SOD peroxidase activity resulted in forming a major peak product

($C_5H_4N_4O_2S$; 182.9981 m/z), which corresponded to 6-sulfoxide-mercaptopurine (6-sulfoxide-MP) as indicated by using LC/MS analysis. 6-MP, but not azathioprine (AZA), significantly decreased $CO_3^{\bullet-}$ radicals as evidenced by using EPR, further indicating that SOD peroxidase activity oxidized 6-MP. Interestingly, SOD activity was protected by 6-MP but not AZA. Lastly, pre-incubation of 6-MP with H_2O_2/HCO_3^- resulted in significantly decreased the cytotoxic effect of 6-MP as judged by the attenuated metabolic activity of a normal (HEK293) cell line and HepG2 cells.

Our overall findings indicate that the combination of drugs with SOD peroxidase-derived $CO_3^{\bullet-}$ radicals appeared to modulate their metabolism and consequently their cytotoxic effect. These findings may have relevance for drugs administered to patients who have chronic inflammation.

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ALS amyotrophic lateral sclerosis

AP-1 activator protein 1

ATP adenosine triphosphate

AZA azathioprine

BAP bicarbonate-activated peroxide

BSA bovine serum albumin

b SOD1 bovine-copper, zinc-superoxide dismutase

CAT catalase

$CO_3^{\bullet-}$ carbonate radical anion

Cu, Zn-SOD copper, zinc-superoxide dismutase

DDC diethyldithiocarbamate

DMEM Dulbecco's modified Eagle's medium

DMPO 5,5-dimethyl-1-pyrroline-N-oxide

DPBS Dulbecco's phosphate-buffered saline

DTPA diethylene triamine pentaacetic acid

Duox dual oxidase relatives

EPR electron paramagnetic resonance

ERK extracellular signal –regulated kinase

HCO_3^- bicarbonate

HCO_4^- peroxymonocarbonate

HEK293 human embryonic kidney 293 cells

HepG2 human hepatocellular carcinoma cells

H_2O_2 hydrogen peroxide

HIF-1 α hypoxia-inducible factor-1 alpha

HGRT hypoxanthine-guanine phosphoribosyl transferase

$HO^{\bullet}$ hydroxyl radical

$HO_2^{\bullet}$ hydroperoxyl radical

HOCl hypochlorous acid

HRP horseradish peroxidase

h SOD1 human-copper, zinc-superoxide dismutase

IMPDH inosine monophosphate dehydrogenase

G/GO glucose/glucose oxidase

GSH glutathione

GSSG glutathione dimer (oxidized)

GST glutathione s-transferase

MMP-3 matrix metalloproteinase-3

MNP 2-methyl-2-nitrosopropane

Mn-SOD manganese-superoxide dismutase

6-MP 6-mercaptopurine

MPO myeloperoxidase

MPSO sulfoxide-mercaptopurine

NF-kB nuclear factor kappa-light-chain enhancer of activated B cells

NO nitric oxide

NOXs NADPH oxidases

NQO NAD(P)H:Quinone oxidoreductase

4-OH-PBZ 4-hydroxy-phenylbutazone

4-OOH-PBZ 4-hydroperoxy-phenylbutazone

$ONOO^-$ peroxynitrite

$ONOOCO_2^-$ nitroperoxymonocarbonate

O_2 oxygen

$^1O_2$ singlet oxygen

$O_2^{\bullet-}$ superoxide anion radicals

O_3 ozone

PBZ phenylbutazone

PGHS prostaglandin H synthase

PGs prostaglandins

PPARγ peroxisome proliferator-activated receptor gamma

PTEN phosphatase and tensin homolog deleted on chromosome 10

$RO^\bullet$ alkoxyl radical

Rb retinoblastoma

$ROO^\bullet$ peroxyl radical

ROOH organic hydroperoxide

ROS reactive oxygen species

$RSOO^\bullet$ thiyl peroxyl radical

-SH nucleophilic thiol

SOD1 copper,zinc-superoxide dismutase (intracellular)

SOD2 manganese-superoxide dismutase

SOD3 copper,zinc-superoxide dismutase (extrtracellular)

SOD-OH oxidant bound-superoxide dismutase

SODs superoxide dismutases

SP1 specificity protein 1

STAT3 signal transducer and activator of transcription 3

SULF sulfinpyrazone

6-TG 6-thioguanine

6-TGN 6- thioguanine nucleotide

TIMP thioinosine monophosphate

TPMT thiopurine methyl transferase

6-TUA 6-thiouric acid

6-TX 6-thioxanthine

XO xanthine oxidase

V volts

Chapter 1

Introduction

1.1 Reactive oxygen species (ROS)

When an oxygen molecule has one or more unpaired electrons in its outermost valence shell is considered as a free oxygen radical, which is highly reactive (ROS). Broadly, there are two types of ROS: (i) the free oxygen radicals that have a single unpaired electron in their outer molecular orbital such as superoxide anion radicals ($O_2^{\bullet-}$), hydroxyl radical ($HO^{\bullet}$), peroxyl radicals ($ROO^{\bullet}$), alkoxyl radical ($RO^{\bullet}$), and thiyl peroxyl radical ($RSOO^{\bullet}$), and (ii) the nonradical ROS are chemically highly reactive and consequently can be converted to radical ROS including hydrogen peroxide (H_2O_2), singlet oxygen ($^1O_2$), ozone (O_3), organic hydroperoxide (ROOH) and hypochlorous acid (HOCl). Accumulation of ROS in normal cells leads to the oxidation of macromolecules, including nucleic acids, proteins, and lipids [1]. However, ROS accumulation in tumors contributes to tumorgenesis and tumor progression [2]. Thus, it is intriguing to understand how ROS are generated.

1.1.1 Sources of ROS

ROS are generated intracellularly through multiple mechanisms from several locations, including mitochondria, peroxisomes, endoplasmic reticulum, and cell membrane (NADPH oxidase complex). Also, ROS can be formed by phagosomes within specialized cells of the immune system used for pathogen killing [2-4]. Extracellularly, ROS can be formed from pollutants, tobacco, smoke, drugs, xenobiotics, or radiation. Namely, $O_2^{\bullet-}$, H_2O_2 and $HO^{\bullet}$are the most prominent ROS in living system and are the most well studied in cancer [3,23].

The mitochondria are the organelle for electron transport chain in cells in which the electrons are transferred from NADH and $FADH_2$ along a controlled redox pathway

that ends in the four-electron reduction of O_2 to H_2O during respiratory ATP synthesis. However, the chance of electron leakage is eminent during ATP synthesis that culminates in the reduction of oxygen to superoxide anion radicals, which are dismutated spontaneously or catalyzed by the presence of superoxide dismutase enzymes (SODs) into hydrogen peroxide (H_2O_2) [5]. The outer membrane of the mitochondria facilitates the leakage of $O_2^{\bullet-}$ and H_2O_2 into the cytoplasm through the mitochondrial permeability transition pore [5,6].

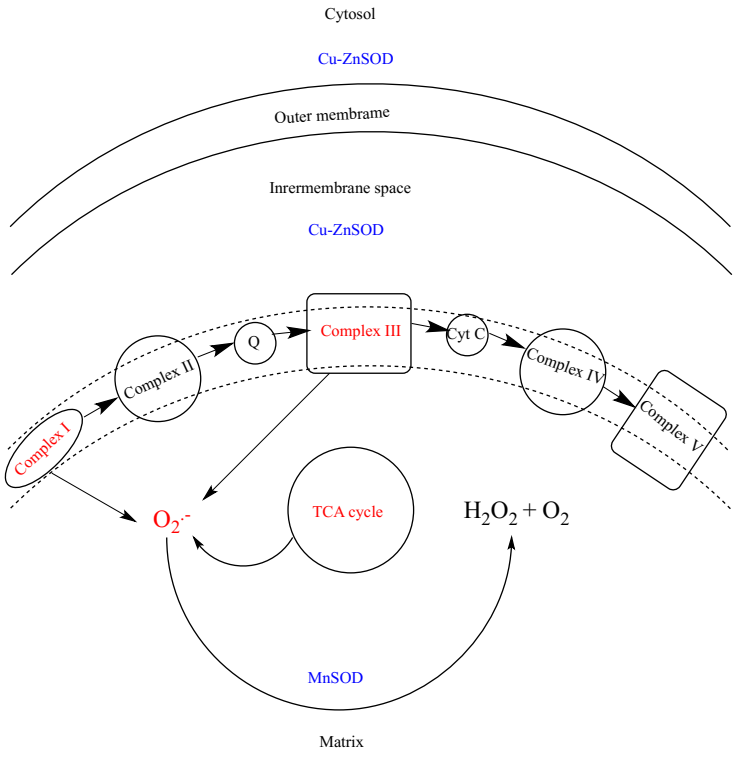

Figure 1.1 The main sites of generating $O_2^{\bullet-}$ in the mitochondrial electron transport chain. The iron-sulfur clusters of complex I, complex III, and tricarboxylic acid cycle (TCA) are the major sites of generating $O_2^{\bullet-}$ that dismutation by manganese-superoxide dismutase (MnSOD) and copper,zinc superoxide dismutase (Cu-ZnSOD) into H_2O_2 and O_2.

The oxidative protein folding and disulfide bond formation are the primary sources of ROS in the endoplasmic reticulum [3]. The glycoprotein endoplasmic reticulum oxidoreductin 1 has an ability to catalyze the vast majority of protein disulfide formation via oxidizing thioredoxin protein disulfide isomerase, leading to introduce disulfide bonds into protein targets through a thiol-disulfide exchange. Endoplasmic reticulum oxidoreductin1 utilized oxygen as a two-electron acceptor, culminating in forming one equivalent of H_2O_2 for each disulfide bond formation catalyzed. Also, NADPH oxidase (Nox4) in endoplasmic reticulum has an ability to generate H_2O_2 from O_2 by a two-electron reduction [3,7].

Peroxisomes are another major site for generating ROS. It has been shown that approximately 20 % of oxygen consumption and 30 % of H_2O_2 production in rat liver can be mediated by peroxisomes [8,9]. These organelles contain various enzymes that contribute to the generation of H_2O_2, including acetyl-CoA oxidase, urate oxidase, D-amino oxidase, D-aspartate oxidase, L-pipecolic acid oxidase, L-α-hydroxy acid oxidase, polyamine oxidase, and xanthine oxidase [9]. The FAD prosthetic group in xanthine oxidase has an important role in the reduction of oxygen to $O_2^{\bullet -}$ and H_2O_2 to $HO^{\bullet}$ [10].

NADPH oxidases (NOXs) and their dual oxidase relatives (Duox), which are localized to various cellular membranes, are another major source of ROS. NOX or phagocyte oxidase is known as a complex protein that consists of $gp91^{phox}$, $p47^{phox}$ $p67^{phox}$, $p22^{phox}$, and Rac. This complex protein located in the plasma membrane has an ability to convert cytoplasmic NADPH into $NADP^+$ by liberating two electrons

and one proton; subsequently, these electrons are transported via the plasma membrane and reduce the oxygen molecules, yielding superoxide radicals [3,10,11].

$$NADPH + 2O_2 \longrightarrow NADP^+ + 2O_2^{\cdot -} + H^+ \quad (1.1)$$

A defect in NADPH oxidase as exemplified by chronic granulomatous disease highlights the importance of ROS production in the immune system. It has been demonstrated that mutations in genes encoding in any one of four essential subunits of the complex ($gp91^{phox}$, $p47^{phox}$ $p67^{phox}$, and $p22^{phox}$) could result in diminished $O_2^{\bullet -}$ production, that may explain why patients who have chronic granulomatous disease are subject to recurrent bacterial and fungal infections; the role of NOX enzymes in the innate immune system has been reviewed [12,13]. Recently, it has been shown that NOX4 contributes to tumor angiogenesis by stabilizing HIF-1α protein expression [14]. Also, Cheng et al. showed that metformin at low concentrations (< 1 mM) could result in decreased ROS levels below the requirement of the human pancreatic cancer cells for survival; however, the anti-survival effect of metformin can be partially reversed by the overexpression of NOX4 protein [15], suggesting that the concentrations of ROS and type of cancer cells appear to show the complexity of ROS in cancer survival and apoptosis. Although the exact mechanism by which metformin decreases NOX4 protein expression has not been explored [15], still these results may suggest that NOX inhibitor could be a therapeutic approach in treating or combating tumor growth.

The main sites of generating ROS in cancer cells is depicted in Figure 1.2 [3]. As we discussed above, mitochondria, peroxisomes, endoplasmic reticulum, and plasma membrane are the main locales of generating $O_2^{\bullet -}$ that dismutation by either Mn-

superoxide dismutase in the mitochondrial matrix or Cu,Zn-superoxide dismutase that localized in both the intermembrane space of mitochondria or in the cytosol, leading to H_2O_2 formation. The latter product is detoxified by catalase or glutathione peroxidase into H_2O. However, accumulation of H_2O_2, which is a highly diffusible oxygen species, could be utilized by myeloperoxidase with the presence of Cl^- to form HOCl, which is a strong oxidant molecule that can oxidize macromolecules, or can be converted by trace metals into a highly reactive radical, $HO^{\bullet}$ (the reduction potential of $HO^{\bullet}$, H^+-H_2O couple is 2.31 V) [3,16] (discussed further in 1.3).

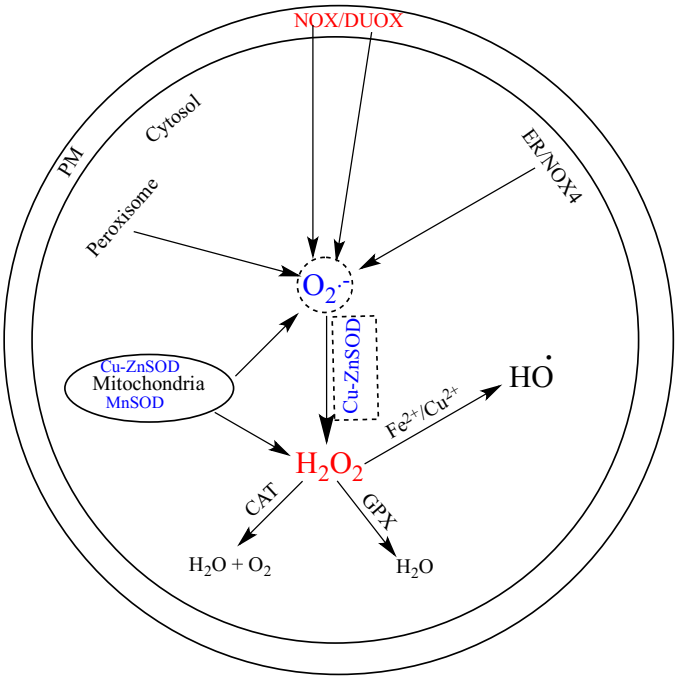

Figure 1.2 The main sources of generating ROS inside a cancer cell. The antioxidant system of the cells, including Mn-SOD, manganese-superoxide dismutase; Cu-ZnSOD, copper-zinc superoxide dismutase; CAT, catalase; and GPX, glutathione peroxidase, protects the cells from excess ROS. (PM, plasma membrane; ER, endoplasmic Reticulum).

1.1.2 Role of ROS in cellular transformation, and tumor cell death

Several studies have shown that free radicals have a pivotal role in cell signaling pathway. For instance, the generation of ROS inside a cancer cell could, in turn, activate transcription factors such as NF-kB, AP-1, HIF-1 α, PPARγ, PTEN, Rb, STAT3, and Sp1, suggesting that ROS have critical role in controlling the expression of genes involved in inflammation, cell transformation, proliferation, invasion, angiogenesis, metastasis, and tumor cell death [17-20].

Cellular transformation. The loss-of-function mutation in tumor suppressor genes, as well as the gain-of- function mutation in oncogenes, can lead to malignant transformation [3]. The transformation of fibroblasts with Rac and Ras was associated with superoxide production; however, this effect could be repressed by antioxidants [3]. It has been shown that hexavalent chromium exposure transformed nontumorigenic lung epithelial cells into malignant cells through stabilization of the cell survival B-cell lymphoma-2 protein, but the overexpression of SOD1, SOD2, and catalase (CAT) could abrogate this transformation [3]. Radiskey et al. observed that treatment with matrix metalloproteinase (MMP)-3, a stromal enzyme that is upregulated in many breast tumor cells, produced increases in cellular ROS via stimulating the expression of Rac1b that lead to induce DNA damage, genomic instability, and the transformation of mouse mammary epithelial cells into malignant cells [21]. Taken together, transformed cells appear to have greater ROS levels than normal cells do and the overexpression of antioxidant enzymes could abrogate this transformation that may suggest that both specific cells and the concentrations of ROS appear to play an important role in cellular transformation.

Tumor cell death. Apoptosis and necrosis are the major pathways by which a cancer cell can die. Apoptosis, a programmed cell death, can be initiated via death receptor (extrinsic pathway) or by mitochondria (intrinsic pathway). ROS are associated with activating both extrinsic and intrinsic pathways of apoptosis [22]. In the extrinsic pathway or death receptor pathway, there are three major known receptors that include Fas (CD95), TNF receptor, and TNF-related apoptosis-inducing ligand (TRAIL) receptors [23]. Death receptors are activated by ligands that include TNF-α, Fas ligand, and TRAIL. The activation of apoptosis signal-regulating kinase1 (ASK1) and proteasomal degradation of $FLIP_L$ by ROS result in the activation of JNK, culminating in apoptosis [3,24]. Wang et al. showed that Fas ligand-induced NADPH oxidase resulted in the generation of $O_2^{\bullet-}$, which is enzymatically or non-enzymatically dismutated to H_2O_2 that mediates the down-regulation of FLIP (a suppressor of Fas death signaling) by ubiquitin-proteosomal degradation [25]. In the intrinsic pathway, ROS trigger the opening of the permeability transition pore complex on the mitochondrial membrane via activating pore-destabilizing proteins as well as inhibiting stabilizing proteins, leading to cytochrome c release, apoptosome formation, and caspases activation [22,26].

The massive levels of ROS in cancer cells may lead to necrotic cell death. It has been shown that the levels of ROS in cancer cells, such as prostate cancer and hepatoma cells could play an essential role in switching from apoptotic to necrotic cell death [3,23]. Also, it was found that 8-nitrocaffeine and its analog could induce necrotic cell death in leukemia cells in ROS-dependent mechanism [3]. On the other hand, the administration of exogenous ROS as well as intracellular ROS produced by

exogenous stimuli may result in the enhanced proliferation of numerous cancer cells. For example, the proliferation of hepatoma cells was found to be enhanced by exogenous administration of H_2O_2 through increasing protein kinase B and extracellular signal-regulated kinase (ERK) activities [27], suggesting that different levels of ROS could activate different signaling pathways.

1.2 Antioxidant enzymes (SOD, catalase)

The cells that can survive in oxygen-rich environments require an effective defense system against ROS. An imbalance between oxidant and antioxidant is considered a major factor in the development diseases such as diabetes, cancer, hypertension, atherosclerosis, inflammation, and premature aging [17,29,50]. In this section, two major antioxidant enzymes in cells: i) superoxide dismutase (SODs) that are the first and most important line of antioxidant enzyme defense systems against ROS, namely $O_2^{\bullet-}$ and ii) catalase that is responsible for detoxifying the cells from H_2O_2 will be covered.

1.2.1 SODs

Superoxide dismutases are the first-line defense against superoxide radicals ($O_2^{\bullet-}$) that are produced during normal intracellular metabolism. SODs catalyze the dismutation of $O_2^{\bullet-}$, to form O_2 and H_2O_2. (eq. 1.2). SODs catalyze the elimination of $O_2^{\bullet-}$ at a rate constant of approximately $1.6x10^9$ $M^{-1}S^{-1}$ at neutral pH [28]. Although superoxide radicals are spontaneously dismutated (k= 10^5 $M^{-1}S^{-1}$), these reaction rates are increased about 10,000 times with the presence of SODs. It has

been proposed that the bond between copper and the imidazole residue, which bridge the copper and zinc site, is cleaved during the reduction of SOD-Cu(II) by the first $O_2^{\bullet-}$, leading to protonate the Zn-imidazolate. The bridge would simultaneously be reestablished during the reaction of the second $O_2^{\bullet-}$ with SOD-Cu (I), resulting in SOD-Cu (II) and H_2O_2 formation [46].

$$O_2^{\cdot-} + O_2^{\cdot-} \xrightarrow{2H^+} H_2O_2 + O_2 \qquad (1.2)$$

There are three distinct isoforms of SOD that have been identified in mammals. The copper, zinc-superoxide dismutase (Cu, Zn-SOD or SOD1) that is localized to either the intermembrane space of mitochondria or intracellular cytoplasmic or nuclear compartments or lysosomes, which has a molecular weight about 32,000 Da; extracellular superoxide dismutase (SOD3), which is present in the extracellular space of cells, also utilizes copper, zinc in its catalytic center but it exists as a homotetramer of molecular weight 135,000 Da with high affinity for heparin; and manganese-superoxide dismutase (Mn-SOD or SOD2) that is located in the matrix of mitochondria with molecular weight of about 23,000 Da [28,29].

1.2.1.1. CuZn-SOD

It has been found that SOD1 gene is localized to chromosome 21 in humans, chromosome 1 in bovine species, and chromosome 16 in the mouse [29]. The mutation of the gene encoded human SOD1 results in Down's syndrome and Trisomy 21 [29]. Some symptoms of Down's syndrome such as the pathological abnormalities of tongue neuromuscular junctions is associated with the increased

level of SOD1 expression. Also, mutations in SOD1 gene could culminate in amyotrophic lateral sclerosis (ALS), which causes degradation of motor neurons in the corticospinal tracts and brain stem [29].

The SOD1 expression is elevated in response to heat shock, UVB, and x-ray radiation as well as in the presence of heavy metals, H_2O_2, nitric oxide, arachidonic acid, ozone and phenobarbital [29]. On the other hand, it has been shown that hypoxia could cause a downregulation of SOD1 in alveolar type II epithelial cells, and lung fibroblasts. Moreover, the transcription of SOD1 could be repressed by using mitomycin C (anticancer drug) in human hepatoma HepG2 cells [29].

1.2.2 Catalase

The enzymatic antioxidant pathway consists of essential two steps: i) the dismutation of superoxide radicals into H_2O_2 by SODs; and ii) the conversion of latter product from step 1 (H_2O_2) by either catalase or glutathione peroxidase into water. Catalases are intracellular oxidoreductase enzymes that are primarily found in highest concentrations in erythrocytes, liver and in the kidney. Peroxisomes are the predominant location for catalase enzymes in tissues, such as the liver. Catalase, a haem-containing enzyme, is a tetrameric protein of 244,000 Da. Human erythrocytes contain 13.7 micromoles of catalase that bound to NADPH [30]. It has been shown that the presence of NADPH could preclude the formation of the inactive form of catalase (iron (IV) oxo-protoporphyrin IX). The main function of catalase is to detoxify H_2O_2 into oxygen molecules and water [30].

$$2H_2O_2 \longrightarrow O_2 + 2H_2O \quad (1.3)$$

The following equations could describe the catalytic activity in catalase that is responsible for H_2O_2 breakdown [30-32]:

$$\text{Catalase } Fe^{+3} + H_2O_2 \longrightarrow \text{Compound I} + H_2O \quad (1.4)$$

$$\text{Compound I} + H_2O_2 \longrightarrow \text{Catalase } Fe^{+3} + H_2O + O_2 \quad (1.5)$$

In both reactions, the catalase enzyme cycles between ferricatalase and Compound I and consequently protects the cells against the damaging effects of H_2O_2. At higher intracellular H_2O_2 concentrations, catalases are more effective in detoxifying or protecting cells compared to glutathione peroxidase 1 (GPX1) and peroxiredoxins, which are responsible for degradation of most H_2O_2 (at low concentrations) [42].

It has been demonstrated that high concentrations of exogenous H_2O_2 (0.01 to 2 M) can result in irreversible inactivation of bovine liver catalase [31]. Also, oxidizing NADPH by H_2O_2 results in $NADP^+$ production and consequently decreased catalase activity. It has been speculated that NADPH could be considered as a cofactor that has a role in generating ROS, dependent on the dose of UVB utilized and the concentration of catalase [30]. It has been reported that catalase could protect HepG2 cells from apoptosis caused by DNA-damaging agents, suggesting that a cell-induced resistance to the effect of anti-cancer drugs could be mediated by the level of catalase [33].

1.3 Role of SODs in modulating cancer progression

It is well-known that a highly oxidized intracellular environment might induce one of the following outcomes: (i) increased proliferation; (ii) adaptation by up-regulation of antioxidant defense; (iii) cell injury; (iv) senescence; (v) cell death [85]. The majority of ROS generated inside cells are oxidizing agents that need to be scavenged by antioxidants (reducing agents). The concentrations of the particular ROS, as well as the form of oxidant and specific cell, could contribute to the complexity of the role of ROS in modulating cell growth. It has been shown that different levels of H_2O_2 can regulate the cellular response. For instance, very low concentrations of H_2O_2 (5-15 μM) can stimulate cellular growth, while increasing the concentrations of H_2O_2 (120-150 μM) may result in temporary growth arrest, followed by an adaptive stress response in which antioxidant proteins expression are induced. However, further increases in H_2O_2 concentration normally reduce cellular growth by activating damage or cell death-related signals [86]. Thus, SODs could play a pivotal role in modulating cell growth through their capability of neutralizing $O_2^{\bullet -}$, yielding H_2O_2 and oxygen.

One of the most well-known characteristics of cancer cells is their fast proliferation. Rabidly, growing tumors results in decreased oxygen (hypoxic condition). The massive production of ROS apparently occurs during reoxygenation of hypoxic tissues as well as when electron transport complexes are in the reduced state [87]. Taken together, ROS levels can be enhanced by hypoxia [87] and normalization of oxygen can be mediated by SODs [46] that could reveal the essential role of SODs in modulating cancer progression.

It has been reported that H_2O_2 (the dismutated product of SODs) may function as a second messenger to regulate growth factor signaling via oxidizing essential cysteine residue in the active site of protein tyrosine phosphatases, suggesting that the levels of H_2O_2 could determine the rate of cell proliferation [88,89]. It is pertinent to point out that $O_2^{\bullet-}$ seems to be innocuous from the reactivity point of view; however, its protonated form ($HO_2^{\bullet}$) is more reactive and can cross cell membranes [90]. The elevation of $O_2^{\bullet-}/HO_2^{\bullet}$ could directly promote cell proliferation and endogenously generated oxidants that can participate in a growth signal through Rac/Ras-MAPK signaling pathway [90]. This may suggest that SODs can play a critical role in activating different signaling pathways.

SODs may serve as tumor suppressive, depending on cell types or disease stages and the levels of ROS. The vast majority of cancer cells show a decrease in the intrinsic levels of antioxidant enzymes including catalase and glutathione peroxidase (GPx), suggesting a potential accumulation of H_2O_2 in cancer cell types as compared to non-transformed cells [37]. It has been postulated that differences in development and metabolism across different organ systems may explain the differences in the levels of antioxidant enzymes across tissue types [37]. It has been hypothesized that there is a strong link between the stable elevation of H_2O_2 and increased SODs activity, which in part may explain the role of SODs in suppressing tumor growth [91,92]. For instance, overexpression of SODs (SOD1 or SOD2) result an in an increased dismutation rate of $O_2^{\bullet-}$, yielding H_2O_2 that indirectly can lead to the formation of other highly-damaging free radicals; for example $HO^{\bullet}$ can be formed, through the well-known iron-catalyzed Fenton reaction [57], leading to oxidize macromolecules

including DNA and proteins [43,44] that subsequently resulting in inhibition tumor growth. Also, it has been shown that SOD2 overexpression could result in increased aconitase activity, decreased intracellular GSH: GSSG ratio and inhibited pyruvate carboxylase enzyme, suggesting that SOD2-mediated ROS could alter cellular metabolic capacity, leading to repress tumor cell proliferation [93]. It has been demonstrated that the effect of SOD2 can be reversed by H_2O_2 as evidenced by expression of mitochondrial catalase [66]. This may suggest that the optimal level of H_2O_2 (a requirement for tumor cell growth) and intracellular antioxidant/redox balance can be altered by SODs, culminating in conditions that are unfavorable for tumor growth. Nevertheless, the differences in modulating cancer progression by activating or inhibiting SODs across cell types may be due, in part, to differences in cell types tested and the levels of dismutating product (H_2O_2).

1.4 The activity of SOD and catalase enzymes in cancer cells

As we discussed above, $O_2^{\bullet -}$ is dismutated by SODs into H_2O_2 that is converted into the harmless product, H_2O, and O_2, by catalase (equations 1.2 and 1.3, respectively). It has been shown that the liver catalase activity is decreased by 22 % in cancer patients compared to cancer-free patients and the weight loss plays an essential role in the human liver catalase depression [34]. There is a significant correlation between the decrease in the catalase activity and the growth of the tumor [34-36], suggesting that potential accumulation of ROS can activate tumor proliferation. It has been demonstrated that SOD2 expression can be down in breast,

esophageal, and pancreatic cancer; however, ovarian, gastric and colorectal cancer showed a high level of SOD2. This discrepancy in SOD2 levels may be due, in part, to the differences in development and metabolism across different organ systems [37,39]. The overexpression of SOD2 results in decreased cell growth of different cell lines such as human melanoma, glioma, prostate carcinoma, and squamous oral carcinoma [37], suggesting that the concentrations of ROS, as well as the form of oxidant and cell types, appear to have a pivotal role in SOD2 tumor suppressive effect [discussed in 1.3]. It has been demonstrated that SOD1 transfected cell lines could lead to an elevation in the ratio of SOD1 activity to glutathione peroxidase activity, which converts H_2O_2 into H_2O, results in higher levels of H_2O_2, yielding to slow proliferation and altered morphology of transfected cells [38]. In addition, the analysis of SOD1 in human primary breast cancers showed an inverse correlation between SIRT3 (the class III NAD^+-dependent histone deacetylase), which appears to be a requirement for the Warburg effect and the reprogramming toward glycolysis; SOD1 was shown to accumulate in both cytoplasm and nucleus of these cells [39]. Furthermore, it has been demonstrated that increased SOD1 activity could suppress the growth of human malignant glioma cells (U118-9 cells) through the accumulation of H_2O_2 or other peroxides, suggesting that SOD1: GPx ratio could play an essential role in cancer growth suppression [40]. The cell growth of hepatocellular cancer cells (HepG2) could be also repressed, to a certain extent, by overexpressing SOD1, as indicated by a reduced number of cells in S phase, decreased clone-forming ability in soft agar, as well as tumor size formed in nude mice [41], suggesting that Cu, Zn-SOD may be a potential tumor suppressor. Nevertheless, the prooxidant-antioxidant

balance within cells could play an important role in developed a therapeutic approach for tumor cells.

1.5 The peroxidase activity of SOD1

In cells, there are different sources of generating superoxide anion radicals, including (but not limited to) xanthine/xanthine oxidase system, NADPH oxidases (NOX) and semiquinone radicals, which are the products that resulted from the reduction of quinone molecules by one electron. The superoxide anion radicals are dismutated or eliminated by SODs, culminating in H_2O_2 [discussed in 1.2]. It is well-known that the site of inflammation is characterized by increased local concentrations of H_2O_2. The H_2O_2 can modulate caspase activity directly but is also involved in the generation of the highly reactive $HO^{\bullet}$ that can oxidize macromolecules at a diffusion-limited rate [43,44].

The study of the reaction between H_2O_2 and bovine or human SOD1 (Cu, Zn-SOD) has been investigated to show the mechanism by which SOD1-derived oxidative stress, (recently reviewed in [45]). Although there are different toxicity mechanisms of H_2O_2, most notably the Fenton reaction which leads to hydroxyl radical ($HO^{\bullet}$) catalyzed by the presence of trace metals, a potential mechanism of H_2O_2 biotoxification could be through a back reaction of SOD [44,45]. Paradoxically, this back reaction results in inactivation and fragmentation of the enzyme; this is known as SOD peroxidase activity [47,48].

Exposure of SOD1 to H_2O_2 results in an irreversible inactivation of the enzyme through forming the bound oxidant, SOD-Cu(II)OH, that is different from $HO^{\bullet}$ [45,49]. The enzyme-bound oxidant, SOD-Cu(II)OH, has an ability to oxidize the

histidine residue in the ligand field of the copper, culminating in inactivating the enzyme [45]. Sankarapandi et al. showed that CuZn-SOD could exert its peroxidase reaction with bound OH not through releasing of $HO^{\bullet}$ radicals from its active site [49]

$$SOD\text{-}Cu(II) + H_2O_2 \longrightarrow SOD\text{-}Cu(I) + O_2^{\cdot -} + 2H^+ \qquad (1.6)$$

$$SOD\text{-}Cu(I) + H_2O_2 \longrightarrow SOD\text{-}Cu(II)\text{-}OH + OH^- \qquad (1.7)$$

Hodgson and Fridovich proposed a mechanism (1.6 and 1.7) in which H_2O_2 could reduce SOD-Cu^{+2} into cuprous (SOD-Cu^{+1}), and the latter product is reoxidized by second H_2O_2, leading to form a strong bound oxidant, SOD-Cu(II)-OH that could react with either bicarbonate or CO_2 to form carbonate anion radical ($CO_3^{\bullet -}$), a diffusible oxidant that has an ability to oxidize macromolecules (discussed further in 1.6) [45]. Although there are several studies that showed the mechanistic pathway in which SOD drives oxidative stress, the study of SOD1 peroxidase activity in oxidizing drugs is still elusive compared to human peroxidases such as MPO and PGHS peroxidase.

1.6 Human peroxidase enzymes (MPO, PGHS)

Peroxidases are heme-containing enzymes, which have an ability to oxidize a variety of xenobiotics via utilizing H_2O_2. The genetics, polymorphism, and evolution, as well as the catalytic site of human peroxidases such as myeloperoxidase, eosinophil peroxidase, uterine peroxidase, lactoperoxidase, salivary peroxidase, thyroid peroxidase and prostaglandin H synthase, have been reviewed [81].

1.6.1 Prostaglandin-endoperoxide H synthase peroxidase

It is well known that prostaglandins (PGs) have a pivotal role in a variety of biological processes, including inflammation. Prostaglandin H synthase (PGHS) consists of two isoforms, PGHS1 that is present at essential constitutive in cells, and PGHS2 that is undetectable in most cells. Evidently, PGHS has a central role in the inflammation process as it contains both cyclooxygenase (COX) and peroxidase activity. PGHS is activated by hydroperoxide such as PGG2, leading to form a compound I that contains a porphyrin radical, which oxidizes the tyrosine residue at position 385 of PGHS1 or at position 371 of PGHS2, culminating in tyrosyl radical. The latter radical attracts one electron from arachidonic acid in order to initiate the radical chain reactions in PG synthesis [82].

Aspirin (acetyl salicylic acid) has analgesic, anti-pyretic and anti-inflammatory actions, and exerts its effect through acetylation of an essential serine at the active sites, namely serine 530 in PGHSP1 and serine 516 in PGHSP2, that result in irreversible inhibition of cyclooxygenase activity, but without affecting the peroxidase activity of PGHS [82]. Marnett et al. showed that chemicals, which were termed by “cosubstrates”, could be oxidized by PGHS to prostaglandins during the oxidation of arachidonic acid, these reactions were termed as “cooxidation” [83]. Ultimately, forming carbon-or sulfur-centered free radicals by oxidizing cosubstrates results in a peroxyl radical formation, which, in turn, can oxidize xenobiotics and drugs, including indoleacetic acid, retinoic acid, sulfate and phenylbutazone [82].

1.6.2 Myeloperoxidase

MPO is present in human neutrophils, monocytes, and to a lesser extent in tissue macrophages. Human neutrophils contain large amounts of MPO as compared to rodents, suggesting that the chance of metabolizing drugs in human is higher than rodents [82]. The major sites in which MPO and H_2O_2 exert their antimicrobial effect are in neutrophils, kupffer cells, and bone marrow $CD34^+$ myeloid progenitor cells [82]. ROS are generated by the activation of neutrophils and monocytes, and MPO catalyzes the formation of HOCl from the H_2O_2-dependent oxidation of chloride ion.

$$\text{MPO Compound I} + Cl^- + H^+ \longrightarrow \text{MPO} + \text{HOCl} \qquad (1.8)$$

Excessive production of HOCl results in tissue damage through oxidizing biomolecules containing thiols, nitrogen compounds or unsaturated double carbon bonds. Also, aromatic amines such as anilines and benzidines undergo a one-electron oxidation by MPO-compound I [82]. Of particular interest is that HOCl can oxidize SOD, leading to oxidative stress and increased damage in inflammation [84].

1.7 Oxidative stress

Oxidative stress is simply defined as an imbalance between the production of free radicals and antioxidant systems. Although the moderate levels of ROS may maintain appropriate redox balance and consequently stimulate cellular proliferation, the overproduction of ROS results in oxidative stress that may inflict severe cellular damage; including DNA adducts formation, lipid peroxidation, and macromolecule oxidation and enzyme inactivation [50,51].

The role of bicarbonate anion (HCO_3) as a mediator of oxidative stress can be traced back to studies carried out using superoxide dismutase (SOD) [52]. The sources of generating $CO_3^{\bullet-}$ is depicted in Figure 1.3. A more prominent role for HCO_3 has been suggested to involve the peroxidase activity of SOD, where H_2O_2 is oxidized in the active site of the enzyme, forming a transient Cu^{2+}-HO species, which then oxidizes HCO_3 to the carbonate radical anion ($CO_3^{\bullet-}$) [45,47,52].

$$\text{SOD-Cu(II)-OH} + HCO_3/CO_2 \longrightarrow \text{SOD-Cu(II)} + CO_3^{\cdot-} + H^+ \qquad (1.9)$$

The $CO_3^{\bullet-}$ can also be produced by homolytic dissociation of nitroperoxymonocarbonate ($ONOOCO_2^-$), which is formed between CO_2 with peroxynitrite ($ONOO^-$). It is believed that under inflammatory conditions the superoxide radicals and nitric oxide ($^{\cdot}NO$) are overproduced by neutrophils and macrophages, and they combined with a rate constant from 6.6 to 19 x 10^9 M^{-1} S^{-1}, to form $ONOO^-$ [53-56].

$$^{\cdot}NO + O_2^{\cdot-} \longrightarrow ONOO^- \qquad (1.10)$$

$$ONOO^- + CO_2 \longrightarrow ONOOCO_2^- \qquad (1.11)$$

$$ONOOCO_2^- \longrightarrow {}^{\cdot}NO_2 + CO_3^{\cdot-} \qquad (1.12)$$

The rate constant of reacting bicarbonate with $HO^{\bullet}$,that is formed by the reaction of H_2O_2 with either $O_2^{\bullet-}$ (Harber-Weiss reaction) or trace metals (Fenton reaction), is k= 8.5x 10^6 $M^{-1}S^{-1}$, that culminate in $CO_3^{\bullet-}$ radicals [57]. It is pertinent to point out that the rate constant of $CO_3^{\bullet-}$ with itself is k= 1.5 x 10^7 M^{-1} S^{-1} [55].

$$\cdot OH + HCO_3^- \longrightarrow H_2O + CO_3\cdot^- \quad (1.13)$$

$$CO_3\cdot^{-} + CO_3\cdot^- \longrightarrow CO_2 + CO_4^{--} \quad (1.14)$$

In spectrometry studies, catalase and bicarbonate catalyzed the oxidation of acetaldehyde by xanthine oxidase, which is known as a key enzyme in purine catabolism, leading to generate $CO_3^{\bullet-}$ [58].

Furthermore, the $CO_3^{\bullet-}$ has been detected with EPR after trapping with DMPO (5,5-dimethyl-1-pyrroline-N-oxide, a spin trap agent) ($k= 2.5 \times 10^6\ M^{-1}\ S^{-1}$) [59]. The $CO_3^{\bullet-}$ does not form a stable spin adduct compared to other radicals such as thiyl radicals, hydroxyl radicals and carbon-centered radicals. The spin adduct usually detected is actually DMPO-OH which is due to the breakdown of the unstable DMPO-OCO2 adduct, or possibly via DMPO oxidation to a radical cation ($DMPO^{\bullet+}$) that ultimately lead to DMPO-OH [59].

Evidence also suggests that the $CO_3^{\bullet-}$ can diffuse to oxidize several targets, including the enzyme that generates it and has been suggested to be involved in familial amyotrophic lateral sclerosis, where a gain of function mutation in SOD may enhance the peroxidase activity producing more oxidative stress [60-62]. The $CO_3^{\bullet-}$ is a diffusible one-electron oxidant ($E^{o'}$ $CO_3^{\bullet-}$, H^+/HCO_3^- = 1.78 V) [55]; it can oxidize many different targets including amino acid residues [55,60]. The rate constant of $CO_3^{\bullet-}$ with tryptophan is $7 \times 10^8\ M^{-1}S^{-1}$, suggesting that tryptophan is a preferred target of $CO_3^{\bullet-}$ [55]. It has been reported that the formation of N-formyl-kynurenine and kynurenine can result from oxidizing tryptophan residue in hSOD1

by $CO_3^{\bullet-}$, that culminate in covalent dimerization of hSOD1 that can be inhibited by tempol, which is known as radical scavenger [63].

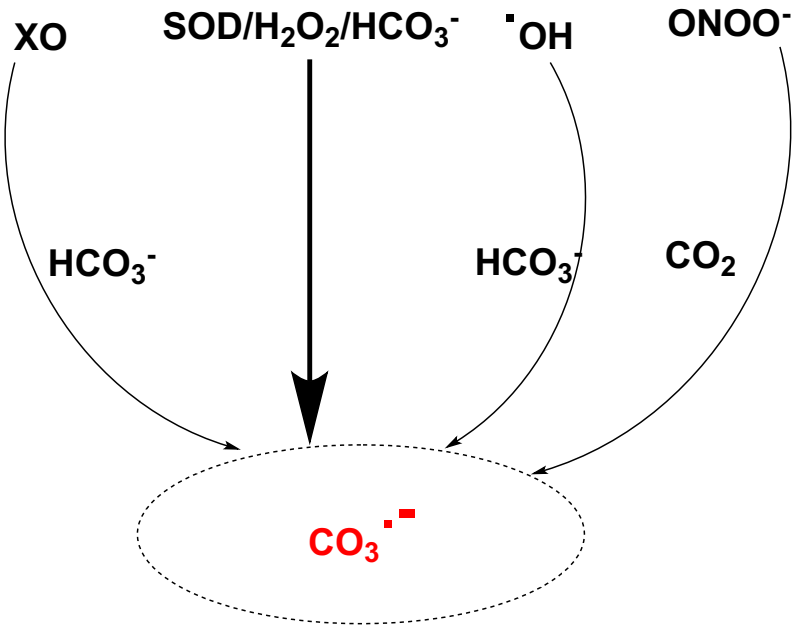

Figure 1.3 The mechanistic pathways of carbonate radical formation. The reaction of bicarbonate with either superoxide dismutase peroxidase activity or xanthine oxidase or hydroxyl radicals, leading to carbonate radical. The reaction of peroxynitrite anion with carbon dioxide is an alternative pathway to carbonate radical formation.

1.8 SOD inhibitors

It has been hypothesized that using exogenous ROS-producing agents may render cancer cells more vulnerable to further oxidative stress through inactivation of SOD [38,64,65]. Huang et al. showed that the inhibition of SOD, in leukemia cells, by 2-methoxyestradiol (2-ME) estrogen metabolites, could result in the accumulation of $O_2^{\bullet-}$, culminating in damaging mitochondrial membranes, release of cytochrome c to cytosol, and consequently activation of apoptotic cascades [67]; however, the exact mechanism by which 2-ME inhibits SOD is still unclear [68]. Also, it has been reported that 2-ME has an ability to slow the proliferation of human ovarian carcinoma cells as compared to normal ovary epithelial cells, suggesting that high levels of ROS can reduce cell proliferation by activating cell-death related signals [65].

There are other compounds such as cyanide, H_2O_2, azide and diethyldithiocarbamate (DDC) that have an ability to inhibit SOD activity. These compounds exert their effect through entering the catalytic site of an enzyme and directly reacting with the metal ions (Mn in SOD2; Cu,Zn in both SOD1 and SOD3). Namely, cyanide and azide ions compete with $O_2^{\bullet-}$at SOD catalytic sites and subsequently repressed SOD activity; however, DDC, at millimolar concentrations, could inactivate the SOD1 by binding to the copper ion and removing it from the enzyme [39,65].

It has been shown that inhibition of SOD1 expression either by shRNA or SOD1 inhibitor, ATN-224, could culminate in decreased proliferation of the lung carcinoma cell line A549, but not the normal bronchial epithelial cells [39]. Glasauer A et al. found that the inhibition of SOD1 by ATN-224 induced non-small-cell lung cancer

death by increased $O_2^{\bullet-}$ formation that diminished glutathione peroxidase activity, and consequently enhanced the formation of H_2O_2 [69].

1.9 Pyrazolidine diketones

In the late 19th century, Emil Fischer identified pyrazolidine-3,5-diones as condensation products of malonic acid and phenylhydrazine. The similarity of pyrazolones biological profile, which were already known as analgesic, anti-inflammatory, and anti-pyretic, was pointed out by Hans Ruhkopf. The structural features of the heterocyclic ring system and side chains in pyrazolidine diketones play important role in modified their biological properties [76,77]. It is well known that phenylbutazone (N, N-diarylpyrazolidin-3,5-dione), which was discovered in 1946, is an analgesic compound and the most prominent drug for osteoarthritis; however, its use has been decreased in the last decades owing to undesired side effects including hepatotoxicity, renal failure, and agranulocytosis. Then, a series of improved agents have been identified, such as oxyphenbutazone, γ-ketophenylbutazone, and sulfinpyrazone.

Pyrazolidine-3,5-dione derivatives have diverse biological activities. They can be considered as the anti-inflammatory therapy for cardiovascular disease, anti-hyperglycemic, anti-tumor, anti-HIV [77,78,80]. Based on a close relationship between inflammation and cancer, pyrazolidine-3,5-dione derivatives showed a significantly inhibitory effect on COX-2 protein and marked inhibition of tumor progression and metastasis [79,80]. Recently, it has been developed potent and

selective anticancer agents, a series of novel 4-substituted 1,2-bis(4-chlorophenyl)-pyrazolidine-3,5-dione derivatives [80]. However, the exact mechanism by which they exert their effect is still elusive.

It is pertinent to point out that phenylbutazone was selected as a candidate of this chemical class to investigate the role of SOD1 peroxidase activity in oxidizing this compound and subsequently the association of SOD1 in its pharmacological as well as toxicological effect.

1.10 Thiopurine derivatives

The thiopurine drugs, 6-mercaptopurine (6-MP), 6-thioguanine (6-TG), and azathioprine (AZA) are widely used to treat a variety of diseases, including inflammatory bowel diseases (IBD) and cancers [70]. 6-MP was initially evaluated as the anti-leukemic drug, and then AZA with its metabolite 6-MP was introduced as the immunosuppressant for organ transplantation. After further clinical observations of thiopurine therapy in IBD, it was found that thiopurines are effective in the treatment of both ulcerative colitis and Crohn's disease [70].

It has been shown that AZA and its metabolite exert their effect in treating IBD through formation 6-thioguanine nucleotide (6-TGN), which incorporates into cellular nucleic acids, that culminate in inhibition of nucleotide and protein synthesis and consequently inhibition of lymphocyte proliferation [73,74]. However, there may be other mechanisms by which thiopurine drugs exert their effects.

6-thiopurines are prodrugs that require bioactivation to exert their cytotoxic effects (Fig. 1.4). It has been shown that 6-thiopurines are converted by hypoxanthine-guanine phosphoribosyl transferase (HGPRT) into 6-thioguanosine 5′-monophosphate (TGMP), which is further metabolized to produce deoxy-6-thioguanosine phosphate 5′-triphosphate. The latter product incorporates into DNA, leading to trigger cell-cycle arrest and apoptosis, this is known as mismatch repair pathway [71]. However, 6-thiopurines can be inactivated by xanthine oxidase (XO) and thiopurine methyl transferase (TPMT) to 6-thiouric acid (6-TUA) and 6-methylmercaptopurine (6-MMP), respectively [72].

It is believed that the inflammation sites are characterized by the generation of ROS that has an ability to oxidize thiol-containing compounds. The oxidation of thiol compounds plays a pivotal role in a variety of biological processes, including the response of antioxidants. It has been reported that levels of CuZn-SOD protein and activity in peripheral blood granulocytes of IBD are low, and also the activities of both catalase and GSH peroxidase1 are suppressed in Crohn's patients [75]. This may suggest that accumulation of ROS could inactivate SOD and subsequently induce SOD peroxidase-derived oxidative stress. Thus, 6-MP was selected as a candidate of the thiopurine drug class to determine the role of SOD peroxidase-derived oxidative stress in oxidizing this compound.

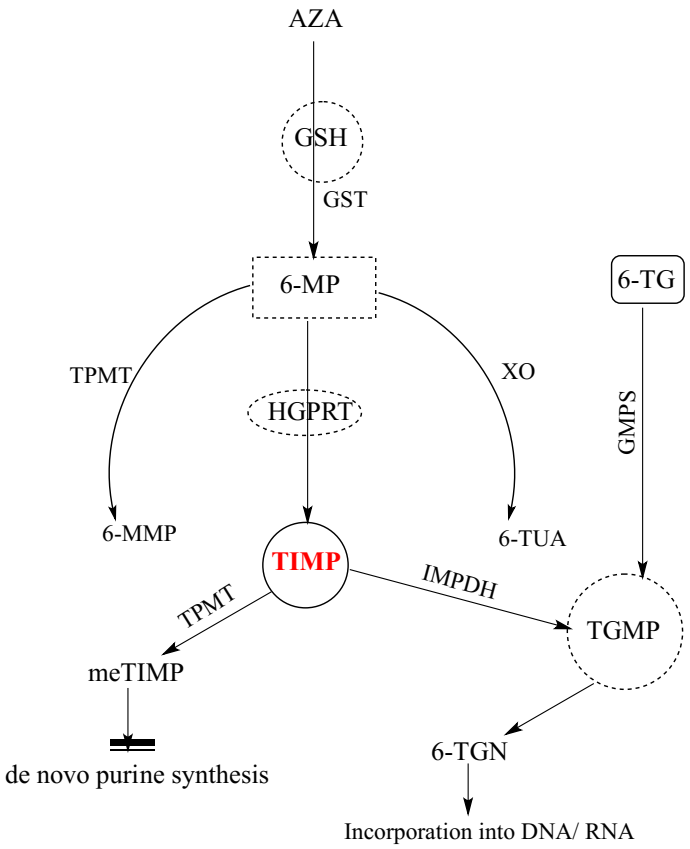

Figure 1.4 Mechanistic pathways of activation and inactivation of 6-thiopurine drugs. AZA, azathioprine; GSH, glutathione; GST, glutathione s-transferase; 6-MP, mercaptopurine; HGRT, hypoxanthine-guanine phosphoribosyl transferase; TIMP, thioinosine monophosphate; IMPDH, inosine monophosphate dehydrogenase; TPMT, thiopurine methyl transferase; XO, xanthine oxidase; meTIMP, methyl thioinosine monophosphate; 6-TG, 6-thioguanine; GMPS, guanosine monophosphate synthase; TGMP, thioguanosine monophosphate, 6-TGN, 6-thioguanine nucleotide; 6-MMP, 6-methyl mercaptopurine; 6-TUA, 6-thiouric acid.

1.11 Rationale, Hypothesis, and objectives

1.10.1 Rationale

Superoxide dismutases (SODs) are enzymes that are present in different locations in cells. In mammalian cells, there are three isoforms of superoxide dismutases (SODs) that include SOD1 (SOD-CuZn), which is present in the cytoplasm and in the intermembrane space of mitochondria, SOD2 (SOD-Mn) and SOD3 (SOD-CuZn) that are located in the mitochondria and in the extracellular cells, respectively. The main function of these enzymes is to catalyze the dismutation of superoxide anion radicals, to form molecular oxygen (O_2) and hydrogen peroxide (H_2O_2). In mammalian cells, the mitochondrial electron transport chain, the endoplasmic reticulum and NADPH oxidase are the major sites of generating superoxide anion radicals. Fortunately, H_2O_2 is detoxified by catalase and glutathione peroxidase enzyme to form O_2 and water (H_2O). However, the activity of catalase enzyme is very low in liver hepatocellular carcinoma, HepG2 cells [34,94]. The overexpression of CuZn-SOD reduces the growth of HepG2, glioma cells, and pancreatic tumor cells [39-41].

In an enzymatic system, the accumulation of H_2O_2 in the presence of bicarbonate and SOD is a pathway to generate the carbonate radical; this process is known as a peroxidase activity of SOD [45,47,48,49]. In addition, the carbonate radical is also generated by the reaction of peroxynitrite [53-56] with CO_2 and during xanthine oxidase turnover in the presence of bicarbonate [58]. The carbonate radical is a diffusible oxidant radical that can oxidize many amino acids, including tryptophan, tyrosine, and histidine, which may lead to protein aggregation [55,63]. It is known

that drugs that possess a diketone moiety in their structures are likely to act as scavengers against reactive oxygen species [94,95]. And oxidation of cellular thiol-containing compounds is produced by the presence of reactive oxygen species. The effect of SOD-derived $CO_3^{\bullet-}$ (SOD peroxidase activity) on the oxidation and subsequent cytotoxicity of pyrazolidine-3,5-dione derivatives (phenylbutazone) and 6-thiopurine derivatives (6-mercaptopurine) has not been investigated. To that end, this work seeks to answer the following questions: i) could SOD peroxidase activity oxidize these drugs? And ii) what are the biological consequences of this oxidation? In Figure 1.5, these research questions are depicted.

1.11.2 Hypothesis

1- SOD peroxidase-derived $CO_3^{\bullet-}$ will oxidize drugs (PBZ and 6-MP) and subsequently will generate their reactive metabolites.

2- The cytotoxicity of drugs that are oxidized by SOD peroxidase-derived $CO_3^{\bullet-}$ will be modulated.

1.11.3 Objectives

1.11.3.1 Specific aims for chapter 2

- To determine if there is an electron transfer between SOD peroxidase-derived $CO_3^{\bullet-}$ and PBZ.
- To characterize the types of PBZ radicals generated.
- To compare the oxidation of PBZ by peroxidase enzymes to the oxidation PBZ by SOD peroxidase activity.
- To validate the formation of PBZ carbon-centered radicals by using MNP-

spin trap and monitoring oxygen consumption.

- To determine the products of oxidizing PBZ by SOD peroxidase-derived $CO_3^{\bullet-}$.

1.11.3.2 Specific aims for chapter 3

- To determine the effect of DDC (SOD1 inhibitor) on SOD1 peroxidase-derived $CO_3^{\cdot-}$.
- To determine the effect of GSH and sorbic acid on both SOD1 peroxidase-derived $CO_3^{\cdot-}$ and PBZ carbon-centered radicals.
- To demonstrate the effect of PBZ on SOD dismutase activity in the presence or absence of SOD peroxidase system ($SOD/H_2O_2/HCO_3$).
- To study the effect of both GSH and sorbic acid on SOD protein and its activity in the presence of SOD peroxidase system and PBZ.
- To demonstrate the cytotoxic effect of combined PBZ with H_2O_2 in the presence or absence of DDC.
- To determine the synergistic cytotoxic effect of extracellular bovine SOD1 on PBZ/H_2O_2 cytotoxicity.

1.11.3.3 Specific aims for chapter 4

- To determine the effect of bicarbonate-activated peroxide system (BAP) on oxidizing 6-thiopurines.
- To determine the reactive metabolite of oxidizing 6-MP by SOD1 peroxidase-derived $CO_3^{\cdot-}$.

- To study the effect of AZA and its metabolite, 6-MP, on SOD1 peroxidase-driven $CO_3^{\cdot -}$
- To determine the effect of both AZA and 6-MP on bovine SOD1 protein and its dismutase activity.
- To demonstrate the effect of oxidizing 6-MP by BAP on its cytotoxic effect.

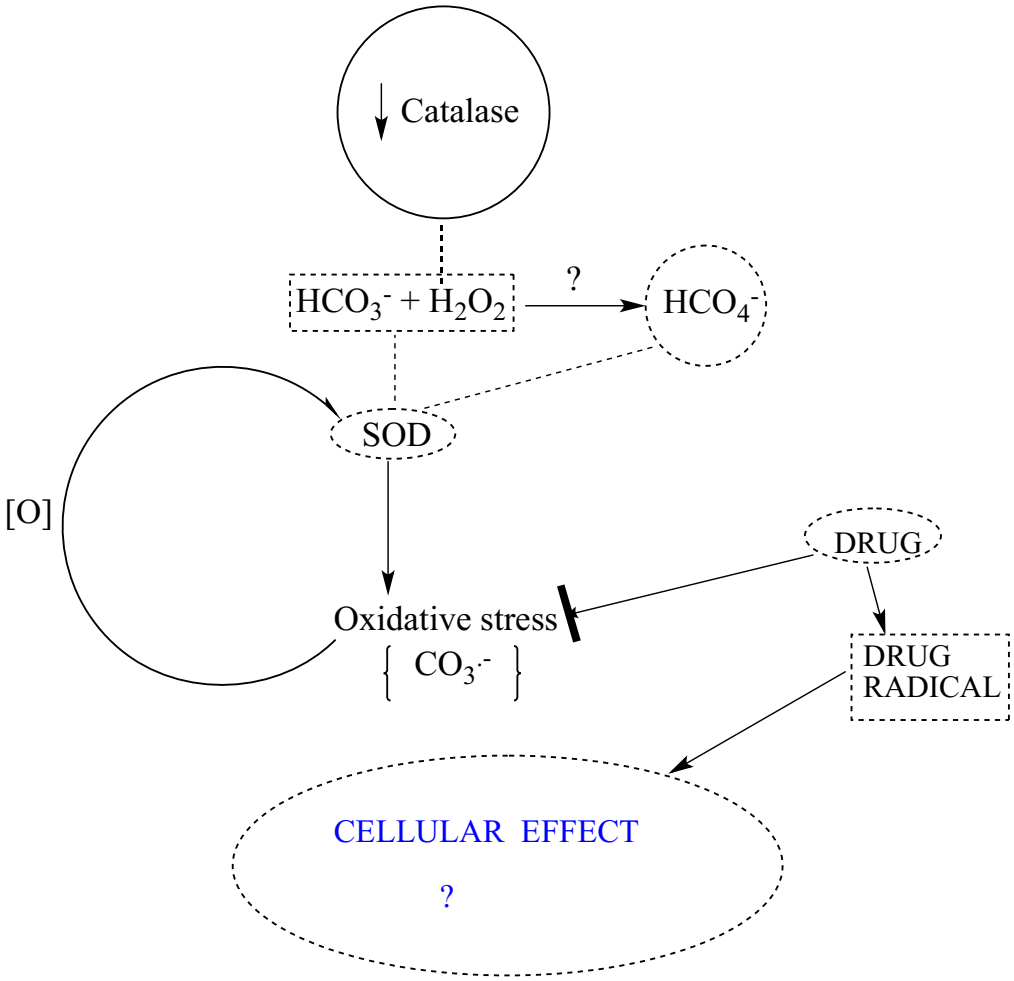

Figure 1.5 Proposed mechanistic pathway of generating reactive drug radical by SOD1-derived carbonate radical. When the catalase activity is very low, there is a chance for accumulation of H_2O_2. SOD1 metabolized H_2O_2, and with the presence of bicarbonate, the carbonate radical will be formed. The latter radical can be scavenged by a drug, leading to generate reactive drug metabolite.

1.12 Strengths and limitations

Fortunately, this is the first study that provides the platform for the role of SOD1 peroxidase-derived oxidative stress in modulating drug metabolism, and understanding the interplay between SOD1 peroxidase activity and drugs could potentially provide a therapeutic approach for selectively killing cancer cells. However, the oxidative stress that results from SOD1, particularly carbonate radical is highly reactive with biological targets. Also, from a methodology standpoint, the $CO_3^{\bullet-}$ is detected indirectly due to the limitation in EPR spin trapping.

1.13 Contribution of colleagues

Chapter 2 was published in *Chemical Research in Toxicology, 28*(7), 1476-1483. All abbreviations, citations, figures and numbering systems in the published work were changed to the format of this thesis. N. Aljuhani envisioned this work, designed and performed experiments, analyzed data and wrote the paper. RM. Whittal performed and discussed LC/MS experiment. S. Khan helped us in the illustrating 3D structure for phenylbutazone/DMPO adduct in EPR experiment. A.G. Siraki edited and approved the final version of the manuscript.

Chapter 3 is being prepared for submission in *Chemical Research in Toxicology*. N. Aljuhani envisioned this work, designed and performed experiments, analyzed data and wrote the paper. Nadine W.M. Commandeur helped us in the quantification of SOD1. S. Khan helped us in editing the reagents. A.G. Siraki edited and approved the final version of the manuscript.

Chapter 4 is being prepared for submission in *Free Radical Biology and Medicine*. N. Aljuhani envisioned this work, designed and performed experiments, analyzed data and wrote the paper. Lindsey Spryut helped us in UV-Vis studies. Bela Reiz and Randy Whittal carried out LC/MS experiment. A.G. Siraki edited and approved the final version of the manuscript.

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Chapter 2

Role of Cu-Zn-superoxide dismutase in oxidizing phenylbutazone (a diketone moiety drugs) [1]

1. A version of this chapter has been published in CRT:

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"Phenylbutazone Oxidation via Cu,Zn-SOD Peroxidase Activity: An EPR study"[1]

…….

2.1 Abstract

We investigated the effect of CuZn-superoxide dismutase (CuZn-SOD)-peroxidase activity on the oxidation of the non-steroidal anti-inflammatory drug phenylbutazone (PBZ). We utilized electron paramagnetic resonance (EPR) spectroscopy to detect free radical intermediates of PBZ, UV-Vis spectrophotometry to monitor PBZ oxidation, oxygen analysis to determine the involvement of C-centered radicals, and LC/MS to determine the resulting metabolites. Using EPR spectroscopy and spin-trapping with 5,5-dimethyl-1-pyrroline-N-oxide (DMPO), we found that the spin adduct of $CO_3^{\bullet-}$ (DMPO/$^{\bullet}$OH) was attenuated with increasing the PBZ concentrations. The resulting PBZ radical, which was assigned as a carbon-centered radical based on computer simulation of hyperfine splitting constants, was trapped by both DMPO and MNP spin traps. Similar to CuZn-SOD-peroxidase activity, an identical PBZ carbon-centered radical was also detected with the presence of both myeloperoxidase (MPO/H_2O_2) and horseradish peroxidase (HRP/H_2O_2). Oxygen analysis revealed depletion in oxygen levels when PBZ was oxidized by SOD peroxidase-activity, further supporting carbon radical formation. In addition, UV-Vis spectra showed that the λ_{max} for PBZ (λ=260 nm) declined in intensity and shifted to a new peak that was similar to the spectrum for 4-hydroxy-PBZ when oxidized by CuZn-SOD-peroxidase activity. LC/MS evidence supported the formation of 4-hydroxy-PBZ when compared to a standard, and 4-hydroperoxy-PBZ was also detected in significant yield. These findings together indicate that carbonate radical, a

product of SOD peroxidase activity, appears to play a role in PBZ metabolism. Interestingly, these results are similar to findings from heme peroxidase enzymes, and this context of this metabolic pathway is discussed in terms of a mechanism for PBZ-induced toxicity.

2.2 Introduction

Phenylbutazone (PBZ) is a non-steroidal anti-inflammatory drug that has powerful analgesic and anti-inflammatory action in the treatment of acute and chronic rheumatoid arthritis; however, its use in human medicine is hampered because of increased risks of agranulocytosis, aplastic anemia, hematemesis, perforation of peptic ulcer, hepatotoxicity, and renal failure [1-3]. PBZ has remained in use for equine medicine, mainly for the treatment of laminitis [4]. However, the aeitiology of PBZ-induced deleterious side effects is not clearly understood.

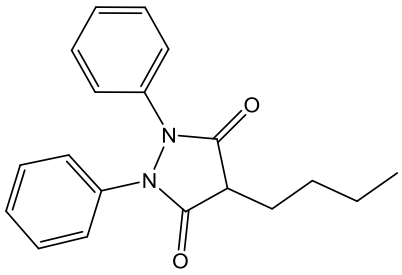
PBZ is a well-studied anti-inflammatory agent. Early studies found that it suppressed the release of lysosomal enzymes which was believed to play a role in joint disease, but PBZ is best known for its ability to inhibit prostaglandin synthesis from arachidonic acid [5,6]. It is well known that prostaglandin H synthase (PHS) plays a pivotal role in the synthesis of hydroxy endoperoxide (PGH_2), which serves as a precursor to many other important biomolecules [7-10]. It has been shown that the hydroperoxidase activity of PHS can also oxidize PBZ to initially generate a carbon-centered radical that reacts with molecular oxygen to form a peroxyl radical and the latter radical, per se, is required to inactivate of the cyclooxygenase activity of PHS [11].

There is consistent evidence that links the formation of PBZ radicals to the presence of peroxidases. For example, horseradish peroxidase (HRP, a plant peroxidase) can catalyze the formation of PBZ radicals that are reported to inactivate α_1-antiproteinase, cholinesterase and creatine kinase [12-16]. Ichihara et al. emphasized that PBZ is likely to act as a scavenger against reactive oxygen species because it possesses a 1,3-diketone moiety in its structure. This suggests that there may yet be other pathways to generate PBZ radicals [17,18].

Superoxide dismutases (SODs) are essential enzymes in cells, which were discovered by McCord and Fridovich [19,20]. Namely, there are three major isoforms of SODs, including SOD1 (CuZn-SOD) that is present in the cytoplasm and in the intermembrane space of mitochondria; SOD2 (MnSOD) and SOD3 (CuZn-SOD) are located in the mitochondria and in the extracellular space, respectively [21,22]. The main function of these enzymes is to protect cells from damage induced by free radicals through their ability to rapidly dismutate superoxide radicals ($O_2^{\bullet -}$) [23]. However, the formation of H_2O_2 that results from the dismutation of $O_2^{\bullet -}$ can result in SOD1 inactivation, yielding a localized hydroxyl radical (SOD-OH) that is a potent oxidant; this process is known as a peroxidase activity of SOD [24-26]. Similar to SOD1, SOD2 also possesses peroxidase activity [27]. In the presence of bicarbonate (HCO_3^-), SOD-OH will oxidize the latter and produce the carbonate radical ($CO_3^{\bullet -}$) [25,27,28]. The redox potential of $CO_3^{\bullet -}$ is 1.78 V ($CO_3^{\bullet -}$, H^+ / HCO_3^-); thus $CO_3^{\bullet -}$ is considered as a diffusible one-electron oxidant that oxidizes many amino acids including tryptophan, tyrosine, and histidine, which may lead to protein aggregation [29,30]. Moreover, CuZn-SOD has been implicated in a broad

range of diseases, such as inflammatory diseases, ischemia-reperfusion injury and cancer [31,32]. However, it is unknown if $CO_3^{\bullet-}$ can oxidize PBZ to generate free radical metabolites. In this study, we hypothesized that $CO_3^{\bullet-}$, a product of CuZn-SOD peroxidase activity, would oxidize PBZ.

A



B

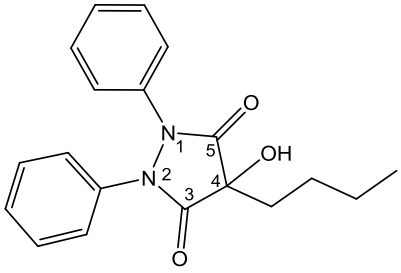


Figure 2.1 Chemical structures of phenylbutazone (A) and 4-hydroxyphenylbutazone (B).

2.3 Experimental procedures

2.3.1 Reagents

PBZ, sodium phosphate monobasic, sodium phosphate dibasic, sodium hydroxide, potassium chloride, magnesium sulphate, D-glucose, hydrogen peroxide (H_2O_2), $NaHCO_3$, diethylene triamine pentaacetic acid (DTPA), 2-methyl-2-nitrosopropane (MNP) and glucose oxidase were purchased from Sigma Aldrich (Oakville, ON). 5,5-Dimethyl-1-pyrroline-N-oxide (DMPO) was purchased from Cedarlane Laboratories and was determined to be of high purity such that vacuum distillation was not required (manufactured by Dojindo Molecular Technologies, Inc., Japan). Superoxide dismutase from bovine erythrocytes (SOD) was purchased from MP Biomedical, LCC (Santa Ana, CA). 4-Hydroxyphenylbutazone (4-OH-PBZ) was purchased from Toronto Research Chemicals (Toronto, ON).

MNP was prepared by dissolving 4 mg/mL in 0.1 M phosphate buffer, pH 7.4, with mixing the solution overnight in darkness at 34 °C. The solution was kept at -20 °C until required for EPR experimentation. The pH measurements were recorded on a Fisher Scientific Accumet^R pH meter (Waltham, MA).

2.3.2 Electron paramagnetic resonance (EPR) spin trapping

EPR spin trapping experiments were performed using a Bruker Elexys E-500 spectrometer (Billerica, MA) with the following instrument parameters: frequency: 9.81 GHZ, center field: 3497 G, microwave power: 20 mW, modulation amplitude: 0.4 G (for MNP) or 1.0 G (for DMPO), modulation frequency: 100 kHZ, sweep time: 60 s, scan number: 2. After measuring the pH, 200 μL of the reaction was transferred

to a suprasil quartz flat cell and the scan was started immediately. The DMPO/$^{\bullet}$OH, which is indirect evidence for $CO_3^{\bullet-}$, was detected directly in the presence of 1 μM SOD, 33 mM HCO_3^- and either with 25 mM glucose and 7.5U/ 200 μL glucose oxidase or with 250 μM, 500 μM, 1.5 mM and 5 mM H_2O_2. Similar experiments were performed to detect DMPO$^{\bullet}$/PBZ and MNP$^{\bullet}$/PBZ upon addition 50 μM, 150 μM, 450 μM, 1.5 mM and 4.5 mM PBZ. Also, the effect of different concentrations of DDC (50 μM, 150 μM, and 300 μM), sorbic acid (0.5 mM, 1 mM,2 mM,4 mM, and 8 mM), INH (0.250 mM. 0.5 mM, 1mM, 2 mM, and 4 mM), and GSH (5 mM and 10 mM) on both DMPO-OH and DMPO-PBZ adducts were detected. All reactions were performed in 0.1 M phosphate buffer containing 100 μM DTPA, pH 7.4, at room temperature. All reactions were performed in 0.1 M phosphate buffer pH 7.4 containing 100 μM DTPA, at room temperature.

Spectra were simulated using WinSim version 0.98 obtained from the Public EPR Software Tools (National Institute of Environmental Health Sciences, NIH). The 3D atomic coordinates of PBZ$^{\bullet}$/DMPO were used to optimize the energy (force field UFF, 4 steps per updates) by using Avogadro (http://avogadro.cc/wiki/Main_Page http://www.jcheminf.com/content/4/1/17). The energy optimized 3D atomic coordinates of adduct was saved as PDB file. For visualization we used UCSF Chimera developed by RBVI at University of California, San Francisco, USA (http://www.ncbi.nlm.nih.gov/pubmed/15264254).

2.3.3 Spectrophotometric measurements

UV-Vis absorption scans were determined on a Spectra Max M5 Multi-Mode Microplate Reader (Molecular Devices, Sunnyvale CA). Optical spectra were recorded by utilizing a 1 cm path length quartz cuvette. The reactions contained 4 μM SOD, 33 mM HCO_3^-, 1.5 mM H_2O_2 and 50 μM PBZ. All the reactions were carried out in 0.1 M phosphate buffer containing 100 μM DTPA, pH 7.4, at room temperature. All measurements were made in triplicate.

2.3.4 Oxygen Analysis

Oxygen consumption was measured by utilizing a Clark-type oxygen electrode and YSI 5300 biological oxygen monitor (Yellow Spring Inc., Yellow Springs, OH). The data was recorded using a DATAQ interface (Akron, OH). The plot of oxygen consumption was made using MS Excel. The reactions contained 4 μM SOD, 33 mM HCO_3^- and 1.5 mM H_2O_2 with either 1.5 mM PBZ or 1.5 mM 4-OH-PBZ. All the reactions were carried out in 0.1 M phosphate buffer pH 7.4 containing 100 μM DTPA, at room temperature. All measurements were made in triplicate.

2.3.5 Mass Spectrometry

LC/MS was carried out using an Agilent 1260 HPLC with Agilent 6130 mass spectrometer equipped with electrospray as the ionization source. All reaction mixtures contained 4 μM SOD, 33 mM HCO_3^-, 1.5 mM H_2O_2 and 500 μM PBZand were carried out in 0.1 M phosphate buffer containing 100 μM DTPA, pH 7.4, at room temperature. At the required time 2μL of the reaction mixture was injected onto a Phenomenex Kinetex C8, 1.7μm core shell UHPLC column with dimensions

2.1×50mm that was heated to 50°C. The compounds were separated using the following gradient: 2% solvent B to 60% solvent B in 5 min then to 95% solvent B in 0.5 min and hold for 2 min where solvent A is water containing 0.1% formic acid and solvent B is acetonitrile containing 0.1% formic acid at a flow rate of 0.5 mL/min. UV chromatograms were collected at 220nm, 254nm, and 280nm. The mass spectrometer was scanned over the mass range 100 to 1500 in both positive and negative ion mode. Extracted ion chromatograms were generated for each compound and overlaid on the same scale.

2.4 Results

2.4.1 PBZ dependent attenuation of DMPO/$^{\bullet}$OH

The detection of $CO_3^{\bullet-}$ and PBZ radicals was performed using EPR spin trapping with DMPO as the spin trap. In the absence of PBZ, reactions containing DMPO, glucose, glucose oxidase, HCO_3^-, SOD resulted in detection of the DMPO/$^{\bullet}$OH spin adduct (Fig. 2.2 A). With increasing PBZ concentrations, there was a decrease in the signal intensity of DMPO/$^{\bullet}$OH with increasing DMPO/$^{\bullet}$PBZ spin adduct intensity (Fig. 2.2 B-F). The omission of either SOD (Fig. 2.2 G) or glucose oxidase (Fig. 2.2 H) or HCO_3^- (Fig. 2.2 I) from the reaction in Fig. 2.2 F did not reveal any radical adducts.

2.4.2 Effect of 4-OH-PBZ and Simulation of the DMPO/$^{\bullet}$PBZ

DMPO/$^{\bullet}$OH and DMPO/$^{\bullet}$PBZ spin adducts that were produced from the reaction containing DMPO, glucose, glucose oxidase, HCO_3^-, SOD, PBZ and DTPA in phosphate buffer are shown in Fig. 2.3 A. However, when PBZ was replaced with 4-OH-PBZ, DMPO/$^{\bullet}$OH was only detected (Fig. 2.3 B). The spectrum in Fig. 2.3 A was simulated using WinSim version 0.98 obtained from the Public EPR Software Tools (Fig. 2.3 C). The (DMPO/$^{\bullet}$PBZ) spin adduct was simulated by $a^H = 26.1$G, $a^N = 15.1$ G (r=0.98), and the parameters for DMPO/$^{\bullet}$OH were $a^H = a^N = 14.8$ (r =0.98). The structure of the DMPO/$^{\bullet}$PBZ radical adduct is shown below the simulation, and an energy-minimized molecular model (optimized adduct energy = 685.069 kJ/mol) is shown in order to illustrate the effect of the relatively bulky PBZ molecule on the beta hydrogen (shown as H_{β}) of DMPO.

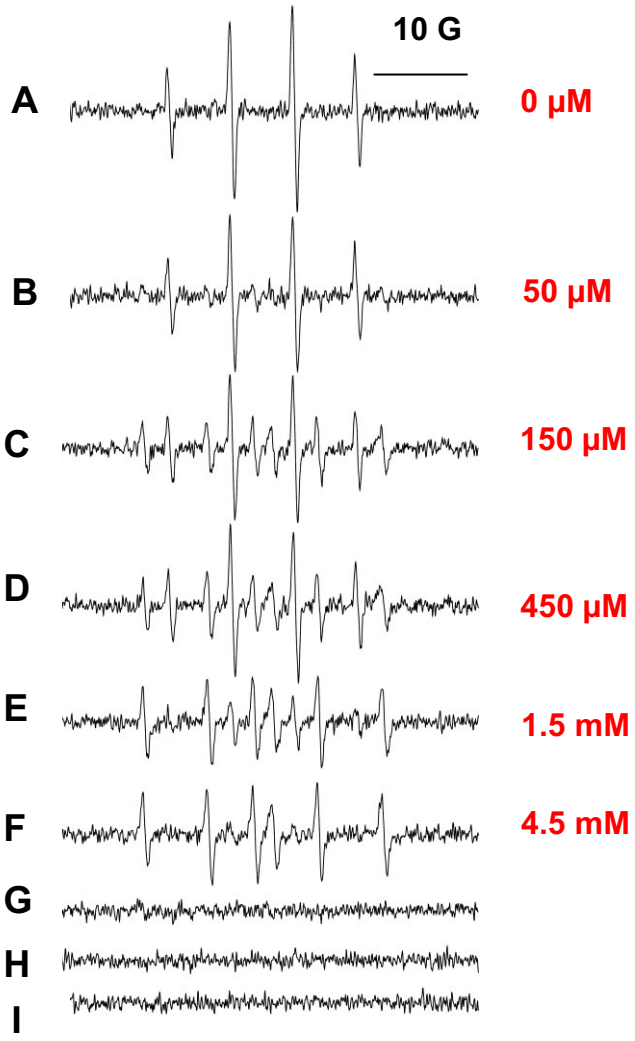


Figure 2.2 Concentration-dependent attenuation of the DMPO/˙OH adduct with increasing PBZ concentration. All reactions were carried out using a 200 µL volume containing 0.1M phosphate buffer containing 100 µM DTPA, pH 7.4, at room temperature. The spectra were generated by the reaction of 100 mM DMPO, 33 mM HCO_3^-, 25 mM glucose, 7.5 U glucose oxidase and 1 µM SOD with 0 µM PBZ (A), 50 µM PBZ (B), 150 µM PBZ (C), 450 µM PBZ (D), 1.5 mM PBZ (E), and 4.5 mM PBZ (F). The omission of either SOD (G) or glucose/glucose oxidase (H) or HCO_3^-(I) from reaction (F) showed no free radical detection in the spectrum. n=3.

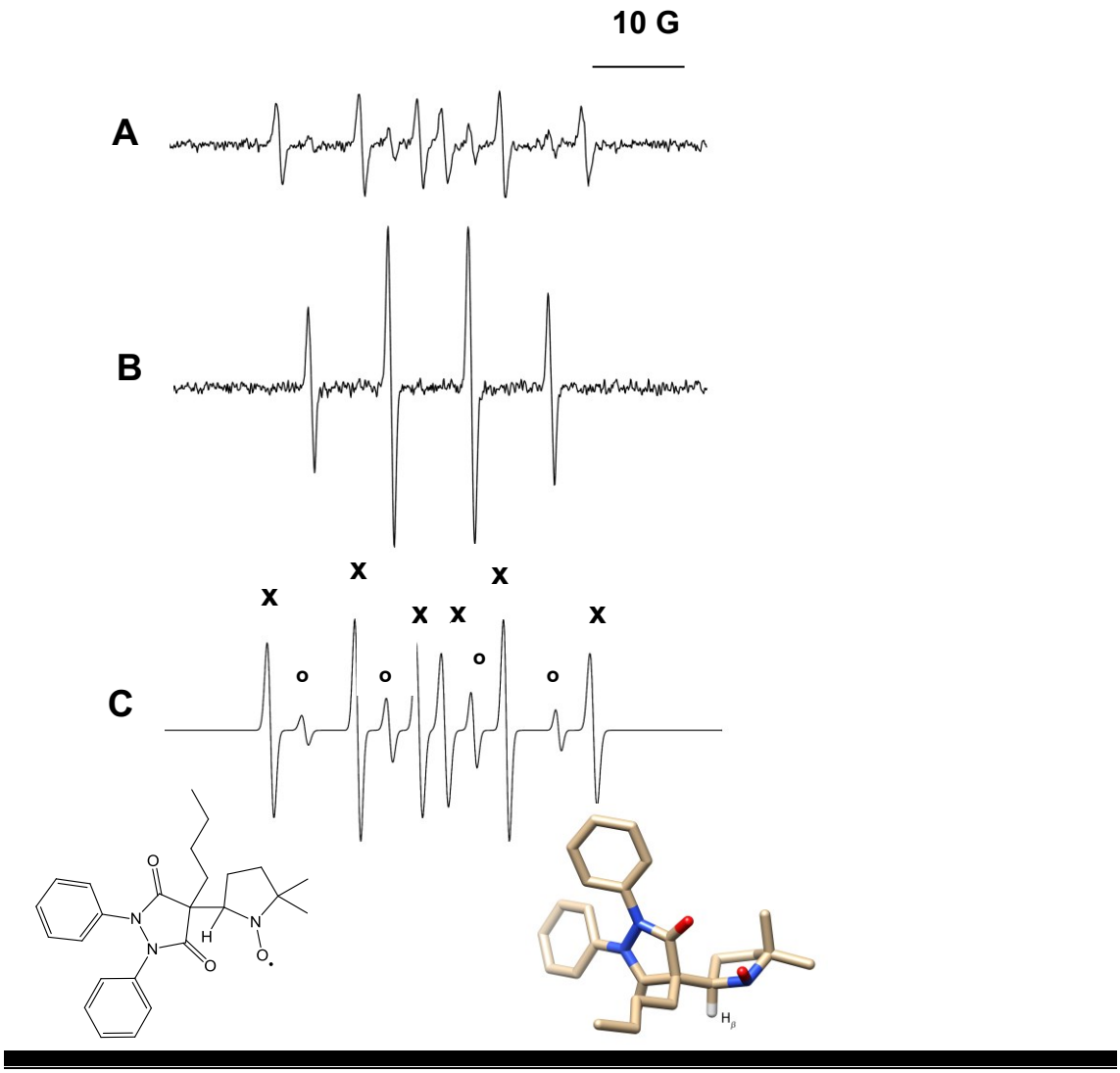


Figure 2.3 The effect of 4-OH-PBZ and characterization of DMPO/$^{\bullet}$PBZ. The spectra from reactions that contained 100 mM DMPO, 33 mM HCO_3^-, 25 mM glucose, 7.5 U glucose oxidase, and 1 μM SOD with either 4.5 mM PBZ (A) or 4.5 mM 4-OH-PBZ (B) are shown. The spectrum shown in (A) was simulated using the following parameters: a^H=14.8 G, a^N=14.8 G (r =0.98) was assigned to DMPO/$^{\bullet}$OH (○) and a^H=26.1 G, a^N=15.1 G (r = 0.99) was assigned to DMPO/$^{\bullet}$PBZ (×) (C)**.** The structure of the DMPO/$^{\bullet}$PBZ adduct is shown below spectrum C, and the energy-minimized molecular model illustrates the effect of PBZ on the beta hydrogen (H_β) of DMPO. n=2

2.4.3 Oxidation of PBZ by MPO

The addition of MPO to the reaction containing DMPO, glucose, glucose oxidase, PBZ, and DTPA in phosphate buffer resulted in DMPO/$^{\bullet}$PBZ spin adducts based on our previous simulation (Fig. 2.4 A); however, no positive spectrum was found when PBZ was replaced by 4-OH-PBZ (Fig. 2.4 B). We did not observe DMPO/$^{\bullet}$PBZ adducts in the absence of either PBZ (Fig. 2.4 C) or MPO (Fig. 2.4 D). Also, there was a detectable, though significantly less intense spectrum, observed when glucose oxidase was excluded from the reaction (Fig. 2.4 E).

2.4.4 Oxidation of PBZ by HRP

These studies were also performed using HRP. The DMPO/$^{\bullet}$PBZ spin adducts was detected upon addition of HRP in reaction containing DMPO, glucose, glucose oxidase and PBZ (Fig. 2.5 A). The spin adduct was significantly attenuated when 4-OH-PBZ was used (Fig. 2.5 B). The signal intensity of DMPO/$^{\bullet}$PBZ was not significant if glucose oxidase was omitted from the reaction (Fig. 2.5 E). In the absence of either PBZ (Fig. 2.5 C) or HRP (Fig. 2.5 D), no radical (spin adduct) spectrum was observed.

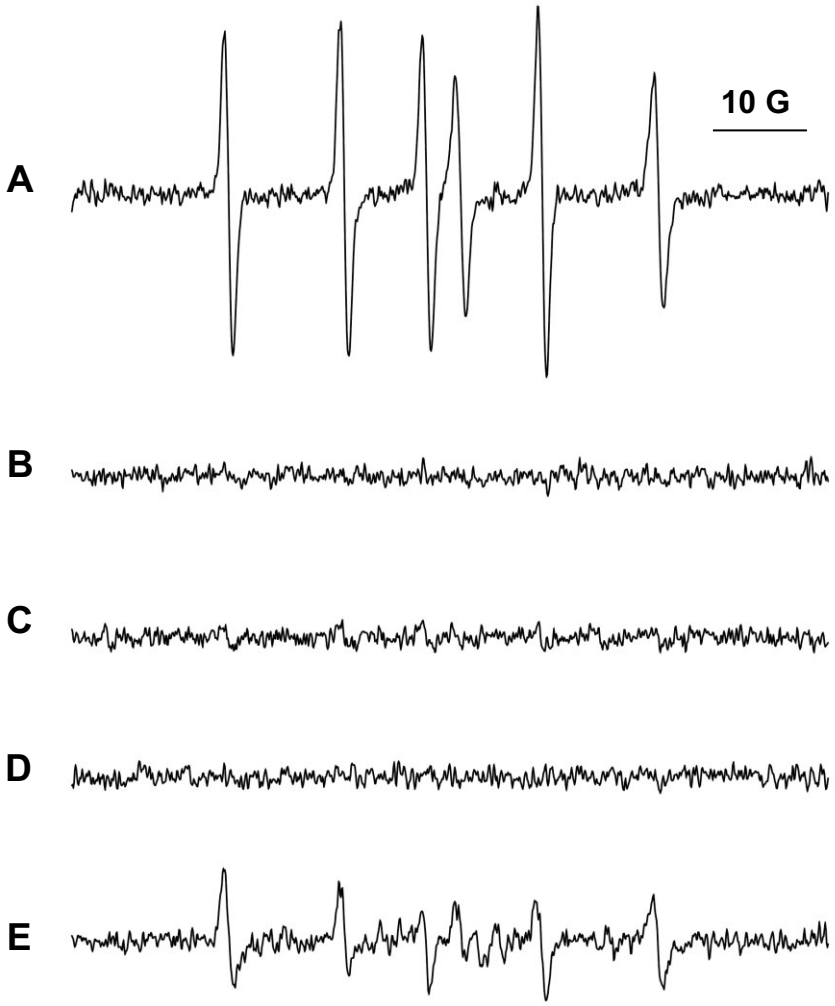


Figure 2.4 Oxidation of PBZ by MPO. The spectra recorded from reactions containing 100 mM DMPO, 25 mM glucose, 7.5 U glucose oxidase, 0.2 nM MPO either with 4.5 mM PBZ (A) or with 4.5 mM 4-OH-PBZ (B). The omissions of either PBZ (C) or MPO (D) or glucose/glucose oxidase (E) from reaction (A) are also shown for comparison. All reactions were carried out in 0.1M phosphate buffer contained 100 μM DTPA, pH 7.4, at room temperature. Instrument parameters clarified in materials and methods. n=3

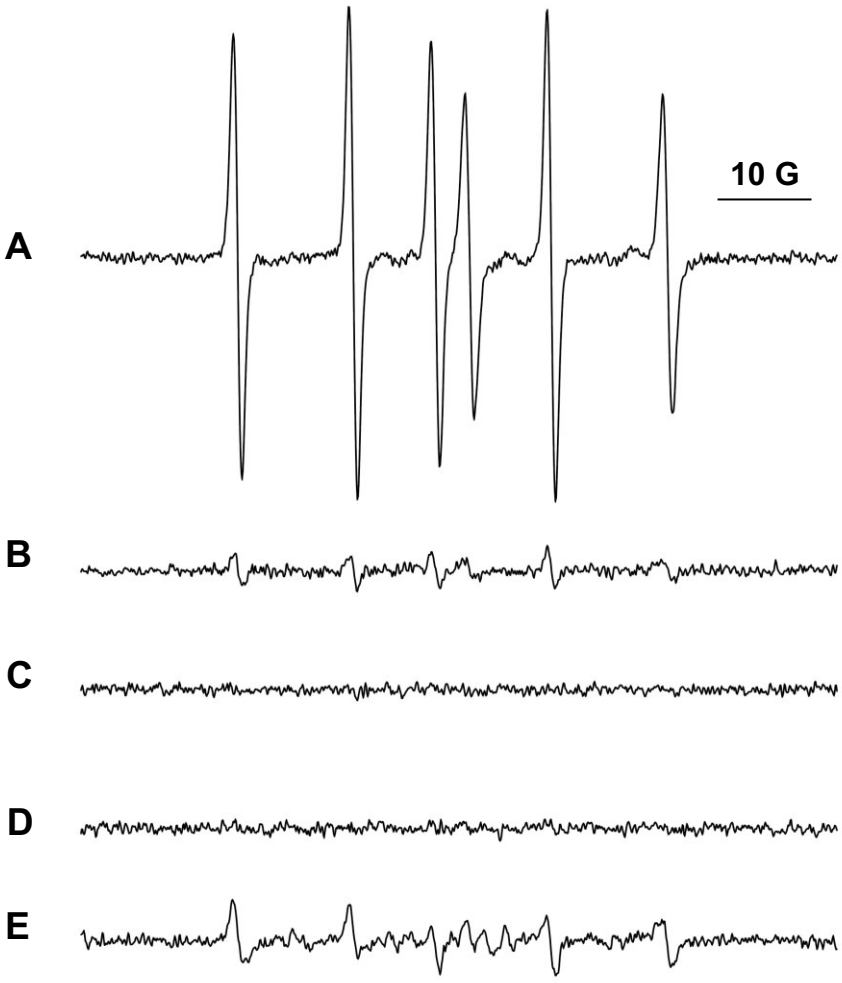


Figure 2.5 Oxidation of PBZ by HRP. The spectra were generated by the reaction of 100 mM DMPO, 25 mM glucose, 7.5 U glucose oxidase, 1 μM HRP either with 4.5 mM PBZ (A) or with 4.5 mM 4-OH-PBZ (B). The omission of either PBZ (C) or HRP (D) or glucose/glucose oxidase (E) from reaction (A) showed no significant spectra. All reactions were carried out in 0.1M phosphate buffer contained 100 μM DTPA, pH 7.4, at room temperature. Reactions were transferred to the EPR flat cell with instrument parameters that are described in materials and methods. n=3

2.4.5. Detection of MNP$^{\bullet}$/PBZ from PBZ oxidation by SOD peroxidase activity

To further confirm the presence of PBZ carbon-centered radicals, we utilized MNP, which is a nitroso spin trap (DMPO is a nitrone spin trap). Indeed, PBZ carbon-centered radicals were detected and trapped using MNP in a reaction containing PBZ, SOD, glucose/glucose oxidase and HCO_3^- (Fig. 2.6 A). In contrast, the omission of PBZ (Fig. 2.6 B), SOD (Fig. 2.6 C), glucose oxidase (Fig. 2.6 D), or HCO_3^- (Fig. 2.6 E) showed spectra that were less intense in comparison to the complete reaction (Fig. 2.6 A); these spectra contained a known photodecomposition product of MNP (Fig. 2.6 B-D) [38].

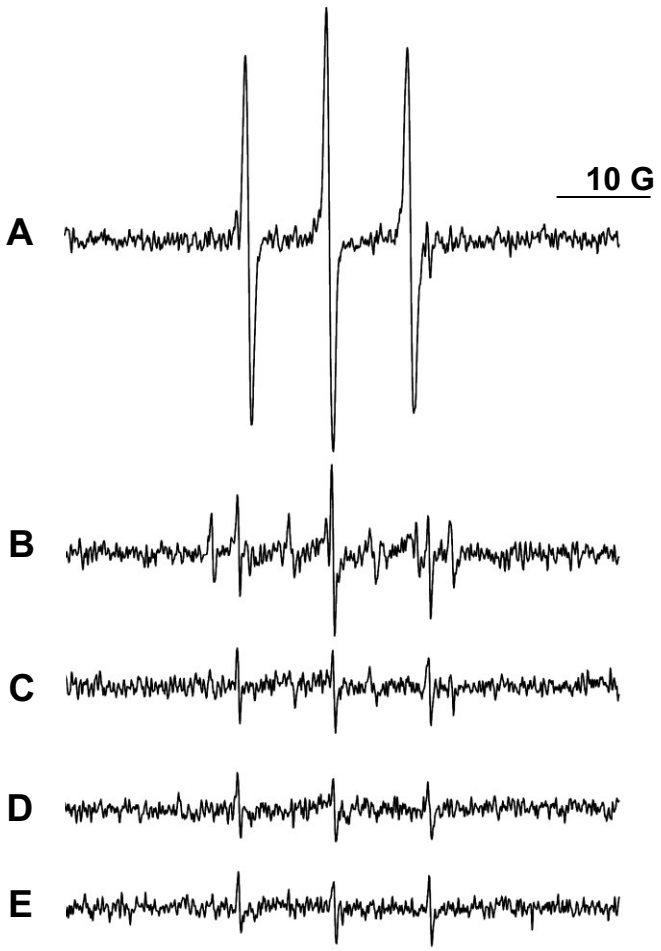


Figure 2.6 Detection of MNP/$^{\bullet}$PBZ from the oxidation of PBZ by SOD peroxidase activity. The spectra were recording after adding 1 μM SOD to the mixture containing 100 mM DMPO, 33 mM HCO_3^-, 25 mM glucose, 7.5 U glucose oxidase and either 4.5 mM PBZ (A) or 0 μM PBZ (B). The resulting spectra from the omission of either SOD (C) or glucose/glucose oxidase (D) or HCO_3^- (E) from reaction (A) are also shown. All reactions were carried out in 0.1M phosphate buffer containing 100 μM DTPA, pH 7.4, at room temperature. Instrument parameters are described in materials and methods. n=3.

2.4.6. SOD peroxidase activity catalyzed oxygen consumption with PBZ

The effect of SOD peroxidase activity on the oxidation of PBZ and its metabolite, 4-OH-PBZ, was further investigated by monitoring oxygen consumption, using a Clark-type oxygen electrode. Oxygen reacts with carbon-centered radicals, making this technique useful for confirmation of EPR studies. As shown in Fig. 2.7, the addition of 4 μM SOD to an aqueous solution of 1.5 mM PBZ, 33 mM HCO_3^- and 1.5 mM H_2O_2 resulted in increased oxygen consumption (decreased oxygen levels); however, in the absence of SOD, or when PBZ was replaced with 4-OH-PBZ, no oxygen consumption was observed.

2.4.7. UV-Vis studies of SOD peroxidase activity-induced PBZ oxidation

The effect of peroxidase activity of SOD on the oxidation of PBZ was studied using UV-Vis spectroscopy. Upon addition of 4 μM SOD to a solution that contained 50 μM PBZ, 33 mM HCO_3^- and 1.5 mM H_2O_2, the intensity of PBZ absorbance (λ_{max} 260 nm) was observed to decrease; however, a peak at 238 nm was produced, suggesting a new product (Fig. 2.8 A). This peak corresponded to the λ_{max} for 4-OH-PBZ, and when SOD was excluded from the reaction, there was no decay in the PBZ peak (Fig. 2.8 B). There was likely another product formed, as evidenced by the shoulder at 232 nm, though this was not further investigated with UV-Vis spectroscopy.

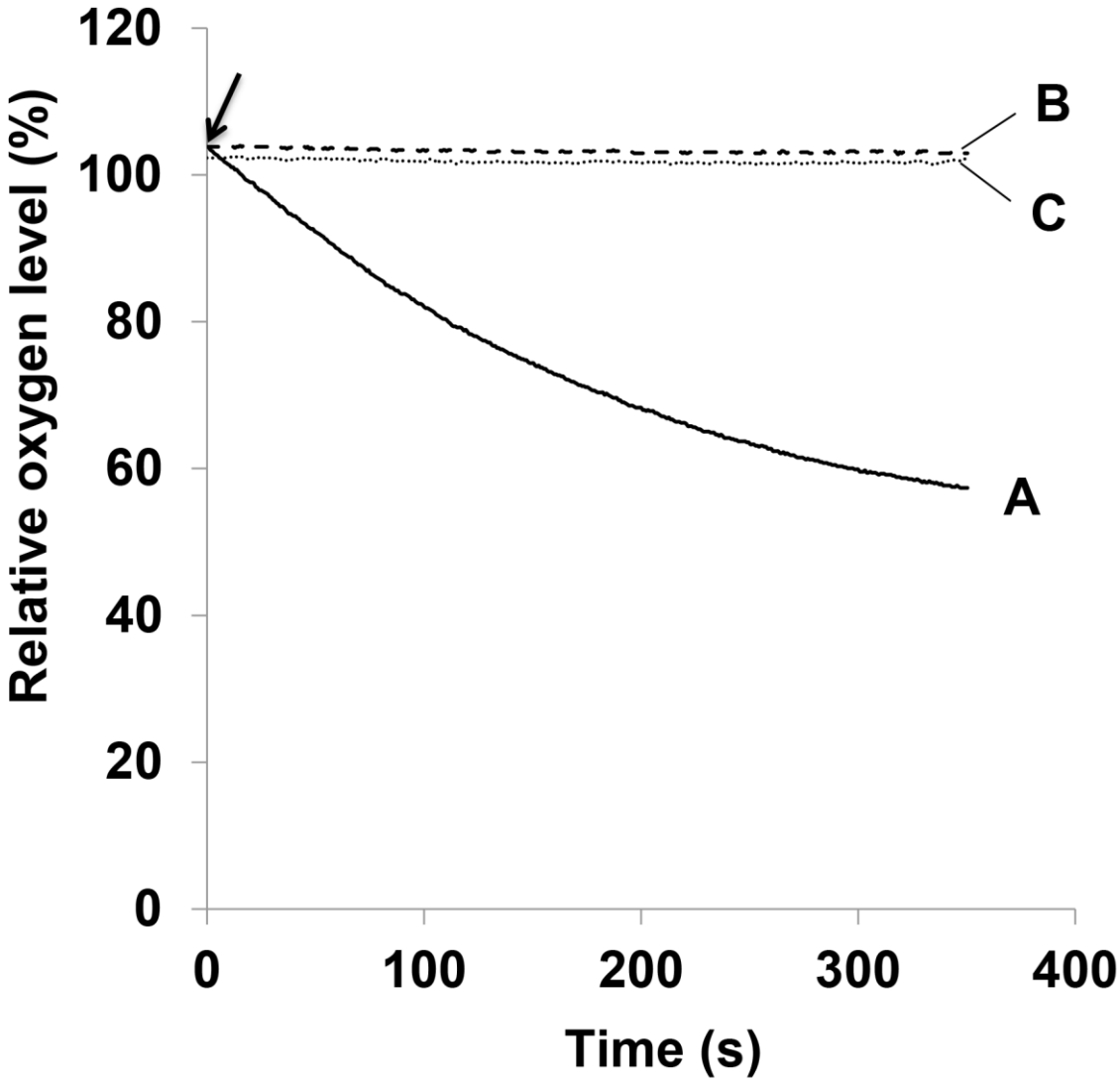


Figure 2.7 SOD peroxidase activity-enhanced oxygen uptake with PBZ, but not 4-OH-PBZ. The reaction was initiated by adding 4 μM SOD at the point indicated (arrow) to the mixture containing 33 mM HCO_3, 1.5 mM H_2O_2, and either 1.5 mM PBZ (A, solid line) or 1.5 mM 4-OH-PBZ (B, dashed line). The omission of SOD from the complete reaction is also shown (C, dotted line). All reactions were carried out in 0.1M phosphate buffer contained 100 μM DTPA, pH 7.4, at room temperature. n=3.

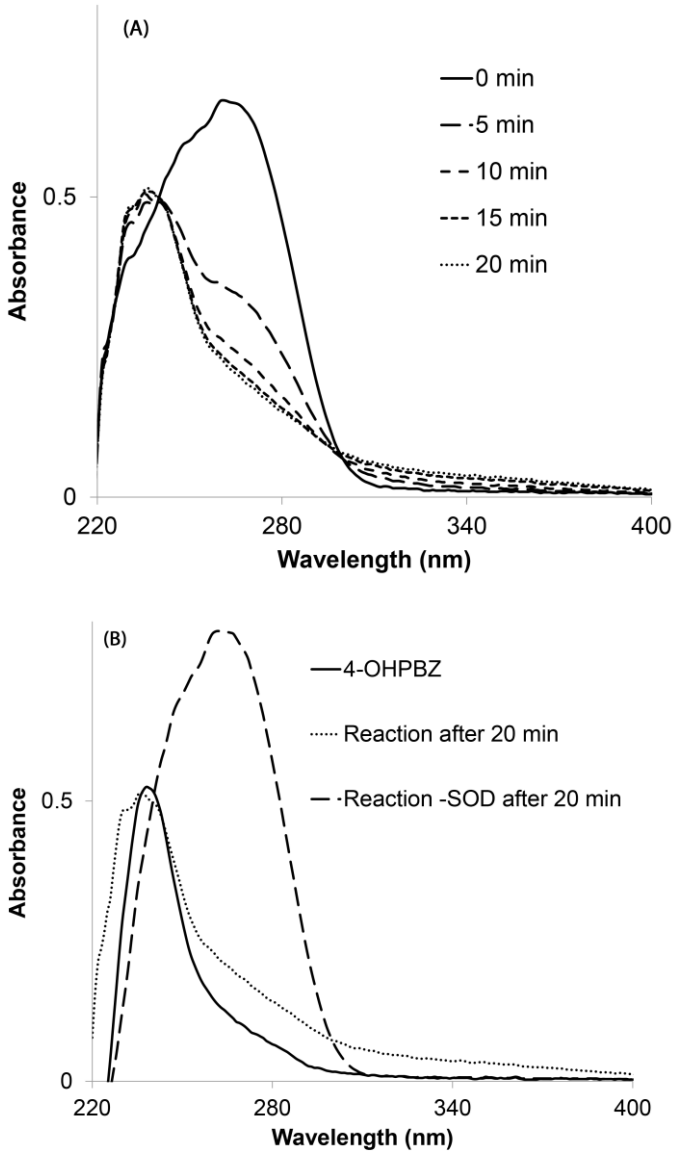


Figure 2.8 UV-Vis spectra demonstrating SOD peroxidase activity-induced PBZ oxidation. A spectrum was recorded from a reaction containing 33 mM HCO_3^-, 1.5 mM H_2O_2, 1 μM SOD and 500 μM PBZ. This reaction spectrum was recorded at 0, 5,10,15, and 20 (A). The omission of SOD from the reaction showed no change in the spectrum of PBZ after 20 min (B). The spectrum of 33 mM HCO_3, 1.5 mM H_2O_2 and 1 μM SOD with 25 μM 4-OH-PBZ is shown after 20 min (B). All reactions were carried out in 0.1M phosphate buffer containing 100 μM DTPA, pH 7.4, at room temperature.

2.4.8 LC/MS studies demonstrated that SOD peroxidase activity produces peroxidase metabolites of PBZ.

The metabolites resulting from PBZ oxidation by SOD peroxidase activity were detected by LC/MS. In the complete reaction (injected at 25 minutes of incubation at room temperature), three major peaks were detected both by HPLC UV at 254nm and by electrospray mass spectrometry at 6.5, 6.2 and 5.9 min (Fig. 2.9). The masses of the peaks corresponded to PBZ (A, m/z = 309.2), 4-hydroperoxy-PBZ (B, m/z = 341.2), and 4-OH-PBZ (C, m/z = 325.2), respectively.

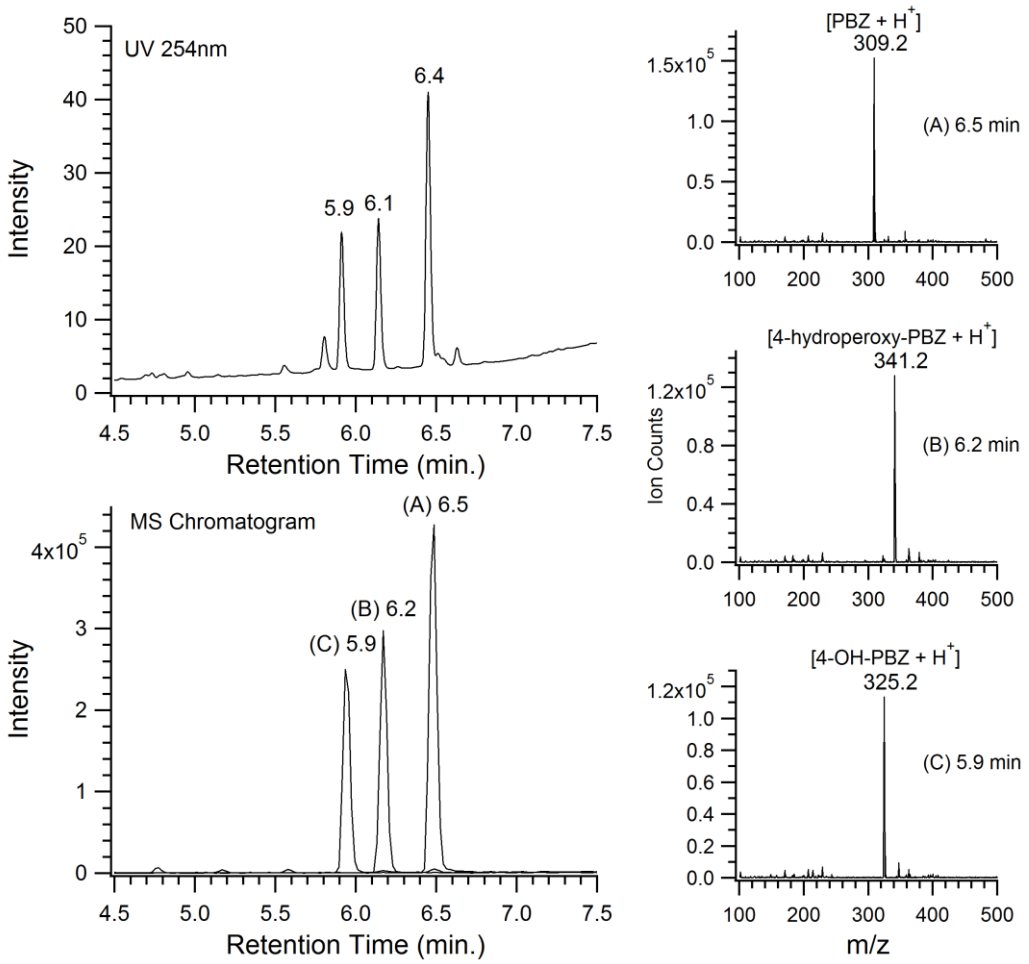


Figure 2.9 LC/MS of PBZ metabolites produced from SOD peroxidase activity demonstrates the formation of PBZ hydroperoxide and 4-OH-PBZ. Reactions were carried out in 0.1 M sodium phosphate buffer pH 7.4 (containing 100 µM DTPA) using a reaction containing 33 mM HCO_3^-, 1.5 mM H_2O_2, 1 µM SOD and 500 µM PBZ. The chromatogram and mass spectrum of the corresponding peaks demonstrated three major compounds: (A, rt=6.5 min) PBZ (m/z 309.2), (B, rt=6.2 min) 4-hydroperoxy-PBZ (m/z 341.2), and (C, rt=5.9 min) 4-OH-PBZ (m/z 325.2).

2.5 Discussion

The current work demonstrates the role of CuZn-SOD peroxidase activity as a catalyst for PBZ oxidation. Figure 2.10 depicts the main findings form this study. SOD peroxidase activity is based on the accumulation of H_2O_2 that would reduce Cu (II) to form Cu (I) in the active site of Cu/Zn-SOD, to in turn react with H_2O_2 to generate a Cu^{+2}- bound $^{\bullet}OH$ [27,29]. However, there has been some controversy among particular substrates (whether HCO_3^- or CO_2) that could result in $CO_3^{\bullet-}$ formation [25].

Electron paramagnetic resonance (EPR) spectroscopy spin trapping is a very sensitive and definitive method to detect short-lived free radicals in biological systems. However, these highly reactive free radicals need to be stabilized using spin-trapping techniques in many cases. In this study, 5,5-dimethyl-1-pyrroline N-oxide (DMPO) and 2-methyl-2-nitrosopropane (MNP) were used as spin traps to indirectly detect $CO_3^{\bullet-}$ as well as PBZ radicals as they provide relatively stable radical adducts. DMPO is quite versatile as it is non-cytotoxic, and offers diverse capabilities in detecting a wide range of radicals, such as carbon-, oxygen-, sulfur-, and nitrogen-centered radicals; MNP is a nitroso spin trap that is relatively cytotoxic and deleterious compared with DMPO, and is mainly restricted to form long lived-adducts with few radicals, e.g., carbon-centered radical [33,34]. Unfortunately, $CO_3^{\bullet-}$ cannot be directly detected through forming a stable spin adduct with DMPO as compared with other reactive radicals, such as O-,C-,S- or N-centered radicals; thus, detecting DMPO/$^{\bullet}OH$ is indirect evidence for trapping $CO_3^{\bullet-}$. Two mechanisms have been proposed for explaining the $CO_3^{\bullet-}$-dependent formation of DMPO/$^{\bullet}OH$: 1) the hydrolysis of an unstable spin adduct, DMPO/$^{\bullet}OCO_2$, could result in forming

DMPO/$^{\bullet}$OH, and 2) oxidation of DMPO could also lead to DMPO/$^{\bullet}$OH [27,28,35]. We observed results consistent with previous reports of DMPO/$^{\bullet}$OH in reactions designed to activate CuZn-SOD-peroxidase activity (SOD/H_2O_2/HCO_3^-) [26,27,28].

In our study, PBZ showed a concentration-dependent attenuation of DMPO/$^{\bullet}$OH (carbonate radical) with an increase in the PBZ radical adduct, DMPO/$^{\bullet}$(PBZ)(Fig. 2 A-F). It is pertinent to point out that DMPO reacts with $CO_3^{\bullet-}$ at a rate constant of 2.5 x 10^6 $M^{-1}S^{-1}$ [36]. In this study, we utilized a large concentration of DMPO (100 mM) to favor trapping $CO_3^{\bullet-}$. However, a PBZ concentration of 150 μM was observed to attenuate the DMPO/$^{\bullet}$OH and form DMPO/$^{\bullet}$(PBZ) suggesting that PBZ most likely has a more favourable rate constant with $CO_3^{\bullet-}$. By taking together the production of $CO_3^{\bullet-}$ via SOD peroxidase activity and the ability of PBZ to scavenge free radicals, forming the PBZ carbon-centred radical could occur through scavenging $CO_3^{\bullet-}$ by PBZ. Thus, the rate of reaction of $CO_3^{\bullet-}$ with PBZ was increased proportionally with PBZ concentration. This study confirmed that CuZn-SOD peroxidase activity is comparable with other studies that showed peroxidases, including PHS hydroperoxidase and HRP, could generate PBZ carbon-centered radicals, although different spin traps were used in those studies [11-16]. Also, we observed both a $CO_3^{\bullet-}$ and PBZ radicals were abrogated in the absence of either SOD, HCO_3^-, or H_2O_2. Previous studies have also found the necessity of HCO_3^- for DMPO/$^{\bullet}$OH detection by SOD/H_2O_2 [26-28]. Therefore, we propose that $CO_3^{\bullet-}$ (DMPO/$^{\bullet}$OH), which is generated by CuZn-SOD peroxidase activity, is able to oxidize PBZ through an electron transfer reaction, yielding secondary PBZ carbon-centered radicals (DMPO/$^{\bullet}$(PBZ)).

To confirm the specific carbon atom involved in these reactions, we utilized 4-OH-PBZ, a commercially available metabolite of PBZ to demonstrate whether the medial carbon atom of the 1,3 diketone moiety in PBZ (C_4 position) is oxidized by $CO_3^{\bullet-}$ (Fig.1 B). In the presence of PBZ, both $CO_3^{\bullet-}$ (DMPO/$^{\bullet}$OH) and PBZ-derived radicals (DMPO/$^{\bullet}$(PBZ)) were trapped and detected. However, $CO_3^{\bullet-}$ (DMPO/$^{\bullet}$OH) was solely detected when PBZ was replaced by 4-OH-PBZ. Thus, the presence of a hydroxyl group at the center carbon of 1,3-diketone moiety in 4-OH-PBZ prevented the reaction with $CO_3^{\bullet-}$. Our finding is consistent with other studies that showed the necessity of the medial carbon atom of a 1,3 diketone moiety in scavenging reactive oxygen species [17,18].

It is noteworthy that the rather large hyperfine splitting computed for the beta-hydrogen associated with the DMPO/$^{\bullet}$(PBZ)adduct has never been reported and is likely the largest beta-hydrogen splitting constant for DMPO (based on accessing the spin-trap database at the NIEHS, http://tools.niehs.nih.gov/stdb/). As shown in the molecular model in Figure 3, the attachment of PBZ at the C_4 position induces the beta-hydrogen to come significantly out of plane relative to the nitroxide. This is consistent with expectations on the hyperfine splitting for a bulky *R* group as described in detail by Janzen [38].

To compare SOD peroxidase metabolism of PBZ with heme peroxidases, we used MPO and HRP. Interestingly, DMPO spin adducts with PBZ have not been previously reported using these peroxidases. For comparison, we found that the carbon-centered radicals (DMPO/$^{\bullet}$(PBZ)) were only formed with PBZ in the presence of MPO/H_2O_2 and with the presence of HRP/H_2O_2, which is in agreement with previous investigations [14-16]. We also found that the carbon-centered radical

could not be detected by using 4-OH-PBZ with both MPO/H_2O_2 and HRP/H_2O_2. These results indicate that $CO_3^{\bullet-}$ as well as MPO and HRP compounds I or II are capable of oxidizing PBZ, but not 4-OH-PBZ.

We carried out further studies by confirming PBZ indeed induced a carbon-centered radical induced by CuZn-SOD peroxidase activity through utilizing the nitroso spin trap, MNP. Utilizing MNP is helpful to determine the type of radical as the reactive radical could directly bind to the nitroso nitrogen atom leading to form a three-line nitroxide radical spectrum [33,34]. As additional lines were not found in the spectrum (indicating that there is no proximal hydrogen atom) this indicates that PBZ is metabolized to a carbon-centered radical by CuZn-SOD peroxidase activity. Our finding is consistent with other studies that showed a generation of PBZ carbon centered radical in HRP/ H_2O_2 system using MNP as the spin trap [11].

Interestingly, we also found that the oxygen uptake was enhanced by the presence of CuZn-SOD peroxidase system in presence of only PBZ; as carbon-centered radicals are known to add oxygen, these findings suggest that CuZn-SOD peroxidase activity oxidized PBZ to form a peroxyl radical, which was derived from a carbon-centered radical reacting with molecular oxygen. These findings are comparable with other studies that showed PBZ carbon-centered radical enhanced O_2 uptake [11,14]. Using UV-Vis spectroscopy, a decrease in absorption of PBZ was observed in the CuZn-SOD peroxidase system. This was accompanied by the formation of a new peak that was similar to 4-OH-PBZ. This suggests that an interaction of PBZ with $CO_3^{\bullet-}$, led to oxidation of PBZ to form 4-OH-PBZ. This finding is consistent with other investigations that showed oxidation of PBZ by leukocytes (myeloperoxidase) resulted in the formation of different metabolites,

including 4-OH-PBZ, 4-hydroperoxyphenylbutazone and 4-chlorophenylbutazone [18]. The final evidence for the metabolic scheme (Scheme 1) was derived from LC/MS studies that confirmed the presence of 4-hydroperoxy-PBZ as well as 4-OH-PBZ, which have been reported as products generated from prostaglandin H synthase peroxidase activity [11].

In summary, the oxidation of PBZ was catalyzed by CuZn-SOD peroxidase activity ($SOD/H_2O_2/HCO_3^-$) through the likely involvement of $CO_3^{\bullet-}$. Oxidation of PBZ by $CO_3^{\bullet-}$ resulted in the formation of a carbon-centered radical, which in turn reacted with molecular oxygen to form 4-hydroperoxy-PBZ; it is possible that the latter would react with $CO_3^{\bullet-}$ to breakdown into 4-OH-PBZ, or that a disproportionation reaction occurred between the initial PBZ carbon radical. We were not able to detect a putative PBZ-OCO_2 intermediate that would breakdown to 4-OH-PBZ (analogous to DMPO). Further studies are required to investigate if CuZn-SOD peroxidase activity is relevant in the cellular environment. It is unknown if this mechanism of PBZ radical formation is causative in the many side effects of PBZ, but a combination of other peroxidase enzymes coupled with CuZn-SOD peroxidase activity could lead to enhanced PBZ radical formation and potential cytotoxicity. Studies for the role of SOD peroxidase-activity in the cytotoxicity of phenylbutazone are currently under investigation.

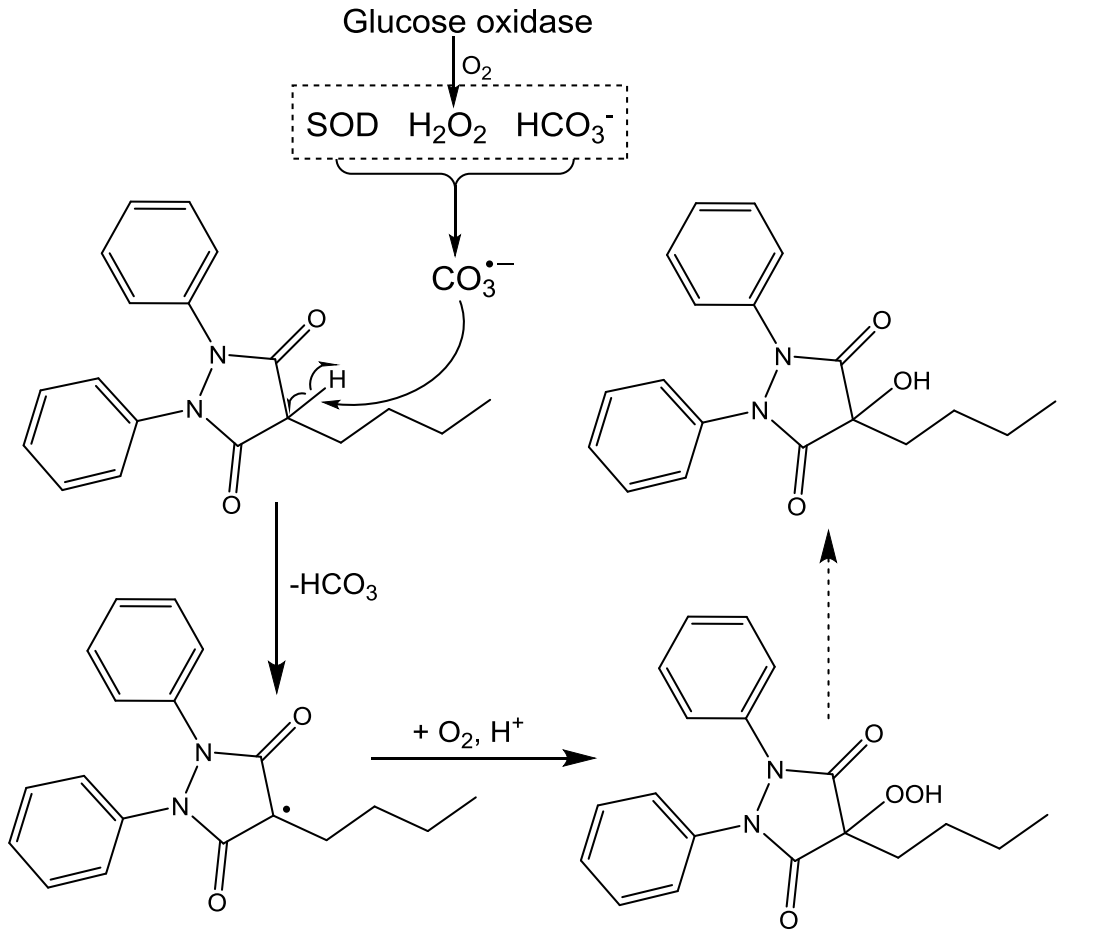


Figure 2.10 Proposed mechanistic pathway of SOD peroxidase activity-induced PBZ oxidation. The carbonate radical produced from SOD peroxidase activity abstracts an electron from PBZ to produce a C-centered radical, most likely at the C-4 carbon. The latter rapidly reacts with oxygen to produce a peroxy radical (a hydroperoxide is shown as this species was detected). It is not completely understood how the hydroperoxide would lead to 4-OH-PBZ (also detected with MS), although it has been proposed that a dismutation reaction would occur between two peroxyl radical metabolites to generate 4-OH-PBZ [11]. All compounds in this scheme were detected either through ESR or LC/MS analyses.

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Chapter 3

Role of Cu,Zn-SOD peroxidase activity in phenylbutazone reactivity

"Role of Cu,Zn-SOD in phenylbutazone reactivity: Toxicological implications in human hepatoma cells (HepG2)"

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3.1 Abstract

We have previously shown that Cu,Zn-superoxide dismutase (SOD1) in the presence of hydrogen peroxide (H_2O_2) and bicarbonate could oxidize phenylbutazone (PBZ), culminating in PBZ-carbon centered radicals. We herein investigated the reactivity of latter radicals towards glutathione (GSH) and sorbic acid and show the role of SOD1 in catalyzing PBZ cytotoxicity using human liver cancer (HepG2) cells. We used electron paramagnetic resonance (EPR) spectroscopy spin trapping using 5,5-dimethylpyrroline-1-oxide (DMPO) to determine the effect of GSH and sorbic acid on PBZ-carbon centered radicals. The metabolic activity and cell viability of HepG2 cells were measured via utilizing both almarBlue and trypan blue assays, respectively. PBZ-carbon centered radicals were attenuated by increasing the concentrations of sorbic acid and GSH. The cytotoxicity of PBZ was synergistically enhanced by the presence of H_2O_2 and SOD1 compared with either H_2O_2 or PBZ used alone. However, the synergistic cytotoxic effect of combined treatment on HepG2 cells was significantly attenuated by the presence of diethyldithiocarbamate (DDC), which was shown to decrease SOD1 activity in HepG2 cells. DMPO/$^{\bullet}$PBZ adducts were detected using intact HepG2 cells; however, the formation of these adducts was significantly decreased by the presence of DDC. Thus, SOD-peroxidase activity appeared to play a role in PBZ-induced toxicity in HepG2 cells.

3.2 Introduction

Reactive oxygen species (ROS) are derived from cellular oxygen metabolism and have both useful and deleterious cellular functions. Chemical instability is one of the manifestations of ROS, which include superoxide anion radicals ($O_2^{\bullet -}$), hydroxyl radical ($HO^{\bullet}$), hydrogen peroxide (H_2O_2), and singlet oxygen ($^1O_2$), leading to damage macromolecules (DNA, proteins, lipids and carbohydrates) [33]. However, redox-buffering systems work as a cellular defense against ROS. Exhausting ROS-buffering capacity can generate oxidative stress - an impaired balance between ROS and antioxidant defences - that are involved in many diseases, including diabetes, cancer, cardiovascular disease, neurodegenerative disorder and arthritis [34].

There are many key systems protecting cells against ROS including superoxide dismutase (SOD), catalase (CAT) and glutathione (GSH). SODs are enzymes that are found in different locations of cells. Copper-zinc-SOD (SOD1) is localized in the cytosol and mitochondrial inter-membrane space; manganese-SOD (SOD2) and copper-zinc-SOD (SOD3) are localized in mitochondria and in the extracellular space, respectively. The main function of SODs is to neutralize $O_2^{\cdot -}$ radicals, a product of cellular metabolism in the cytosol, mitochondria, endoplasmic reticulum and extracellular matrix, culminating in forming H_2O_2 and O_2 [6]. CAT is a peroxisomal enzyme that reduces H_2O_2 to H_2O [35]. GSH acts as a nonspecific scavenger of ROS and subsequently maintains the cysteine residues of proteins in reduced form, and has an ability to stabilize copper in the (Cu^+) oxidation state, culminating in preventing redox cycling and free radical generation [37].

It has been demonstrated that most tumor tissues are characterized by some deficiencies in antioxidant systems; however, the levels of SOD2 are elevated in a few cancer types [38]. Also, diminished GSH levels are found in hepatocellular carcinoma when compared to normal liver tissue [39], and the activity of catalase is low in HepG2 cells as compared to rat hepatocytes [30], suggesting that potential accumulation of H_2O_2.

SOD can also catalyze a reverse reaction known as peroxidase activity, which is initiated by the accumulation of H_2O_2, yielding a potent enzyme-bound oxidant (SOD-OH) [5,6,10,13]. In the presence of a substrate, such as bicarbonate or CO_2, SOD-OH oxidizes either bicarbonate or CO_2 and consequently generates carbonate radical ($CO_3^{\bullet-}$), a diffusible one-electron oxidizing radical [6]. The $CO_3^{\bullet-}$ ($E^{o'}$ $CO_3^{\bullet-}$, H^+/ HCO_3^- = 1.78 V) is capable of oxidizing different amino acids, and consequently causes protein aggregation [24-26].

Phenylbutazone (PBZ) is a non-steroidal anti-inflammatory drug (NSAIDs) that is mainly used to treat acute and chronic osteoarthritis; however, its use was halted because of safety concerns [40-42]. The exact mechanism by which PBZ induced these deleterious side effects has not been known. Also, the mechanism of attenuating these effects remains to be clarified. We have previously shown that $CO_3^{\bullet-}$, produced from SOD1 peroxidase activity, could oxidize PBZ to produce peroxidative metabolites, (4-hydroperoxy phenylbutazone and 4-hydroxy phenylbutazone) [7]. We herein report the role of SOD1 in the cooxidation reaction of PBZ carbon-centered radicals towards sorbic acid (2,4-hexadienoic acid), which is known to be susceptible to nucleophilic attack, and GSH (antioxidant). It has been demonstrated that sorbic acid is highly a reactive compound from the chemical point

of view, as it can react with nitrogen and sulfur-containing nucleophiles [21]. The nucleophilic attack is most likely in the carbon atom of the conjugated diene (a low electron density) [21]. It has been shown that carbon-centered radicals can be considered as electrophiles or nucleophiles, depending on the nature of electron-releasing or electron-withdrawing substituents [43], suggesting that sorbic acid could potentially react with PBZ carbon-centered radicals. Additionally, we evaluated the role of SOD1 in PBZ-induced cytotoxicity via using a human hepatocellular carcinoma (HepG2) cells, as a model for this study.

3.3 Experimental procedures

3.3.1 Reagents and kits

Phenylbutazone (PBZ), human SOD, sodium hydroxide, hydrogen peroxide (H_2O_2), sodium phosphate monobasic, sodium phosphate dibasic, sorbic acid, reduced glutathione, D-glucose, glucose oxidase, sodium bicarbonate ($NaHCO_3$), diethylene triamine pentacetic acid (DTPA) and diethyldithiocarbamate (DDC) were purchased from Sigma-Aldrich Canada Co. (Oakville, ON). 5,5-Dimethyl-1-pyrroline-N-oxide (DMPO) of a high purity (such that vacuum distillation was not required) was purchased from Cedarlane Laboratories Ltd (Burlington, ON). Trypan blue, alamarBlue, trypsin and fetal bovine serum albumin were purchased from Fisher Scientific Company (Ottawa, ON). Bovine SOD was purchased from MP Biomedical, LLC (Santa Ana, CA). SOD Assay Kit-WST was purchased from Dojindo Molecular Technologies, Inc., (Maryland, USA) via Cedarlane Laboratories Ltd. The pH measurements were recorded via utilizing a Fisher Scientific Accumet pH meter (Waltham, MA).

3.3.2 Electron Paramagnetic Resonance (EPR)

Biochemical system-spin trapping EPR experiments with DMPO were performed using a Bruker Elexys E-500 spectrometer with the following instrument parameters: frequency: 9.81 GHz, center field: 3497 G, microwave power: 20 mW, modulation amplitude: 1.0 G, modulation frequency: 100 kHz, sweep time: 60 s. The reaction (200 μL) was transferred to a suprasil quartz flat cell and the scan was started immediately after tuning. The DMPO/$^{\bullet}$OH adduct formation was detected directly in the presence of 1 μM SOD, 33 mM HCO_3^-, and 25 mM glucose + 7.5U glucose oxidase. Similar experiments were performed to detect DMPO/$^{\bullet}$PBZ, a carbon-centered radical, upon addition 1 mM PBZ. The effect of different concentrations of DDC (0.05 mM, 0.150 mM, and 0.300 mM), sorbic acid (0.5 mM, 1 mM, 2 mM, 4 mM, and 8 mM) and GSH (5 mM and 10 mM) on both DMPO/$^{\bullet}$OH and DMPO/$^{\bullet}$PBZ adducts were detected. All reactions were performed in 0.1 M phosphate buffer containing 100 μM DTPA, pH 7.4, at room temperature.

3.3.3 HepG2 cells-spin trapping

DMPO, a non-toxic spin trap, was used for stabilizing $CO_3^{\bullet-}$ and PBZ-carbon centered radicals. HepG2 cells were suspended in a fresh DMEM high glucose medium at a density 10^7 cells/mL. The cell suspension was treated with 50 mM DMPO, 7.5 units of glucose oxidase, and in the presence or absence of 1 mM PBZ. For DDC treatment, the cell suspension was incubated with different concentrations of DDC (10 μM, 50 μM, and 300 μM). The reaction was analyzed by EPR as described above.

3.3.4 Bovine SOD1 dismutase activity measurement

The incubation reaction mixtures were performed as previously described with slight modifications [26]. All experiments were carried out in 100 mM phosphate buffer containing 100 μM DTPA, pH 7.4. The reactions contained 3 μM bSOD1, 1 mM H_2O_2, 33 mM $NaHCO_3$, and different concentration of PBZ (1 μM, 10 μM, 100 μM, and 1 mM), sorbic acid (0.5 mM, 1.5 mM, and 3 mM), and 5 mM GSH. The reaction mixtures were incubated at 37 °C for 3 h. The SOD dismutase activity was measured by using SOD-WST kit according to manufacturer's instructions.

After reaction mixtures were incubated at 37 °C for 3 h, the protein was precipitated by addition of ice-cold acetone (1 mL for each reaction). All the reaction mixtures were kept at -20 °C for 4 h and then were centrifuged at 16000 g for 15 min. The supernatants were discarded and the pellets were washed three times with Dulbecco's phosphate buffer saline (DPBS). Protein concentrations were determined via using BCA protein assay (Biorad laboratories, Mississauga, ON) and aliquots of protein (10 μg) were loaded on the gel for SDS/PAGE (10%). The gels were immersed in fixer solution (50% methanol and 10% acetic acid) for 10 min and followed by silver staining according to company's instructions (BioRad laboratories). We used Image J to relatively quantify of the bands.

3.3.5 Cell culture

The human hepatocellular carcinoma cells (HepG2) were obtained from ATCC, and maintained in Dulbecco's modified Eagle's medium (DMEM; Invitrogen), supplemented with 10% fetal bovine serum (Thermo Scientific), 1% penicillin-

streptomycin, 1% non-essential amino acids. Cells were grown in 75-cm^2 cell culture flasks at 37 °C in a 5% CO_2 humidified atmosphere for 72 h. After washing the cells with DPBS, cells were detached by trypsinization and followed by centrifugation at 300g at 4 °C for 5 min. Cells were seeded at a density of 2.5 x 10^4 in both 96-well and 24-well plates and incubated at 37 °C in humidified incubator, 5% CO_2, for 24 h, prior to the treatments.

3.3.6 PBZ, H_2O_2, and SOD1 treatments

The cells were incubated with different concentrations of PBZ and H_2O_2 at 37 °C, 5% CO_2, for 24 h to determine the concentration response curves. Then, cells were treated with either 300 μM H_2O_2 (a non-cytotoxic concentration) or 1 mM PBZ, a concentration that revealed significant difference from vehicle treatment, or the combination of 300 μM H_2O_2/1mM PBZ for 24 h. For DDC treatments, HepG2 cells were treated with different concentrations of DDC (75 μM, 150 μM, and 300μM) with the presence of combined treatment (300 μM H_2O_2/1mM PBZ) for 24 h. In the presence of combined treatment, cells were treated with either different concentrations of bSOD1 (0.1 μM, 1 μM , and 5 μM) or with 0.15 μM hSOD1 for 24 h.

3.3.7 SOD activity assay in HepG2 cells

HepG2 cells were treated with either deionized water as a control or with 300 μM DDC. All 75 cm^2 flasks were incubated at 37 °C in humidified incubator, 5% CO_2, for 24 h. The supernatant was discarded after centrifugation for 5 min at 300 g and the pellets were suspended in 200 μL RIPA buffer (25 mM Tris-HCl, pH 7.6, 0.05 % SDS, 0.5% sodium deoxycholate, 150 mM NaCl, and 0.5% Triton) and kept on ice

for 20 min. The supernatant was collected for SOD activity after centrifugation of the cell suspension (16000 g for 25 min at 4 °C). SOD assay kit-WST was used to measure SOD activity. By using a logarithmic scale, the dilution ratio at 50 % inhibition of superoxide for each sample was determined.

3.3.8 Cell metabolic activity and viability assay

The metabolic activity of HepG2 cells was measured using alamarBlue a fluorimetric assay. The viable cells catalyzed the reduction of resazurin to resorufin, a highly fluorescent compound. HepG2 cells were treated with either different concentrations of PBZ, H_2O_2, and bSOD1 or combined PBZ/H_2O_2 with the presence of both bovine and human SOD1. After washing cells with DPBS, the cells were incubated with 100 µL of the alamarBlue solution (diluted at 1:10 with DPBS), at 37 °C in humidified incubator, 5% CO_2, for 6 h. Fluorescence intensity was measured using Spectra Max M5 Multi-Mode microplate reader at room temperature via exposure of plates to an excitation wavelength at 570 nm and an emission wavelength 590 nm. HepG2 cell viability was measured by utilizing TC-10 automated cell counter (Bio-Rad laboratories) through mixing cell suspension with trypan blue at a 1:1 ratio. Results were the average of triplicate measurements.

3.3.9 Statistical analysis

Results are expressed as means ± SD. The differences were evaluated by one-way ANOVA or Student's t-test, followed by Student-Newman-Keuls post-hoc test. Values with $^{*}P < 0.05$, $^{**}P < 0.01$ were considered statistically significant differences.

3.4 Results

3.4.1 DDC dependent attenuation of DMPO/$^{\bullet}$OH.

We utilized EPR spectroscopy with DMPO as the spin trap to stabilize and subsequently detect $CO_3^{\bullet-}$. In the presence of the vehicle, DMPO, HCO_3^-, glucose, glucose oxidase, and bSOD1, we could detect DMPO/$^{\bullet}$OH spin adducts (Figure 1 A), which is consistent with other studies [5,6,10,13,14]. We found that there was attenuation of DMPO/$^{\bullet}$OH spin adducts formation by an increase in the concentration of DDC, a strong copper chelating agent (Figure 3.1 B-D).

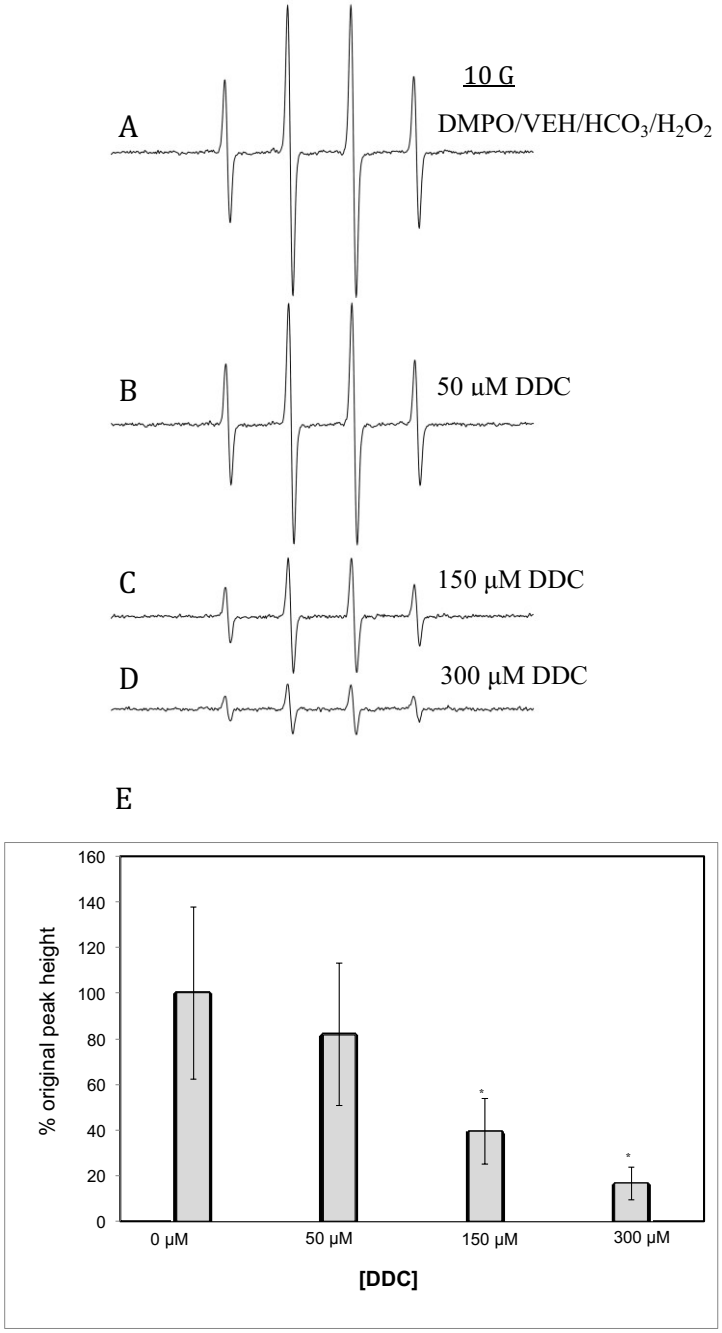

Figure 3.1 Concentration-dependent attenuation of the DMPO/•OH adduct by DDC. EPR spectroscopy was used to record the spectrum resulting from the incubation of 100 mM DMPO, 33 mM HCO_3^-, 25 mM glucose, 7.5 U/200 µL glucose oxidase, and 1 µM bSOD with deionized H_2O as a vehicle (A), 50 µM DDC (B), 150 µM DDC (C), 300 µM DDC (D). The spectra of EPR measurements (**DMPO/•OH** adduct) are shown in (E). All reactions were carried out in 0.1 M

phosphate buffer containing 100 μM DTPA, pH 7.4, at room temperature. Instrument parameters are described in Experimental Procedure; *, $p < 0.05$ compared to vehicle and 50 μM DDC. n=3

3.4.2 Effect of GSH and sorbic acid on DMPO/$^{\bullet}$OH and DMPO/$^{\bullet}$PBZ spin adducts formation.

Figure 3.2 showed that GSH significantly decreased DMPO/$^{\bullet}$OH adduct formation without forming thiyl radical (DMPO/$^{\bullet}$SG) in the absence of PBZ (Figure 3.2 B). However, PBZ carbon-centered radicals (Figure 3.2 C) were attenuated with forming DMPO/$^{\bullet}$SG spin adducts (Figure 3.2 D). An increase in the concentration of GSH resulted in decreased DMPO/$^{\bullet}$PBZ adducts and apparent decay of apparent species (Figure 3.2 E). The absence of bSOD1 showed no free radicals detection in the spectrum (Figure 3.2 F). In Figure 3.3, free radical scavenging of sorbic acid was also determined by EPR. The $CO_3^{\bullet-}$ radicals (Figure 3.3 A) were significantly decreased with the presence of PBZ, leading to PBZ carbon-centered radicals (Figure 3.3 B). In the presence of sorbic acid, the DMPO/$^{\bullet}$PBZ spin adducts dependently decreased by increasing the concentrations of sorbic acid (Figure 3.3 C-G). Only high concentrations of sorbic acid could attenuate DMPO/$^{\bullet}$OH spin adduct formation, as compared to its effect on DMPO/$^{\bullet}$PBZ (Figure 3.4), suggesting that the reaction of $CO_3^{\bullet-}$ with sorbic acid was potentially slow as compared to react $CO_3^{\bullet-}$ with DMPO, and PBZ carbon-centered radical appeared to rapidly react with electrophilic alkenes (2,4-hexadienoic acid).

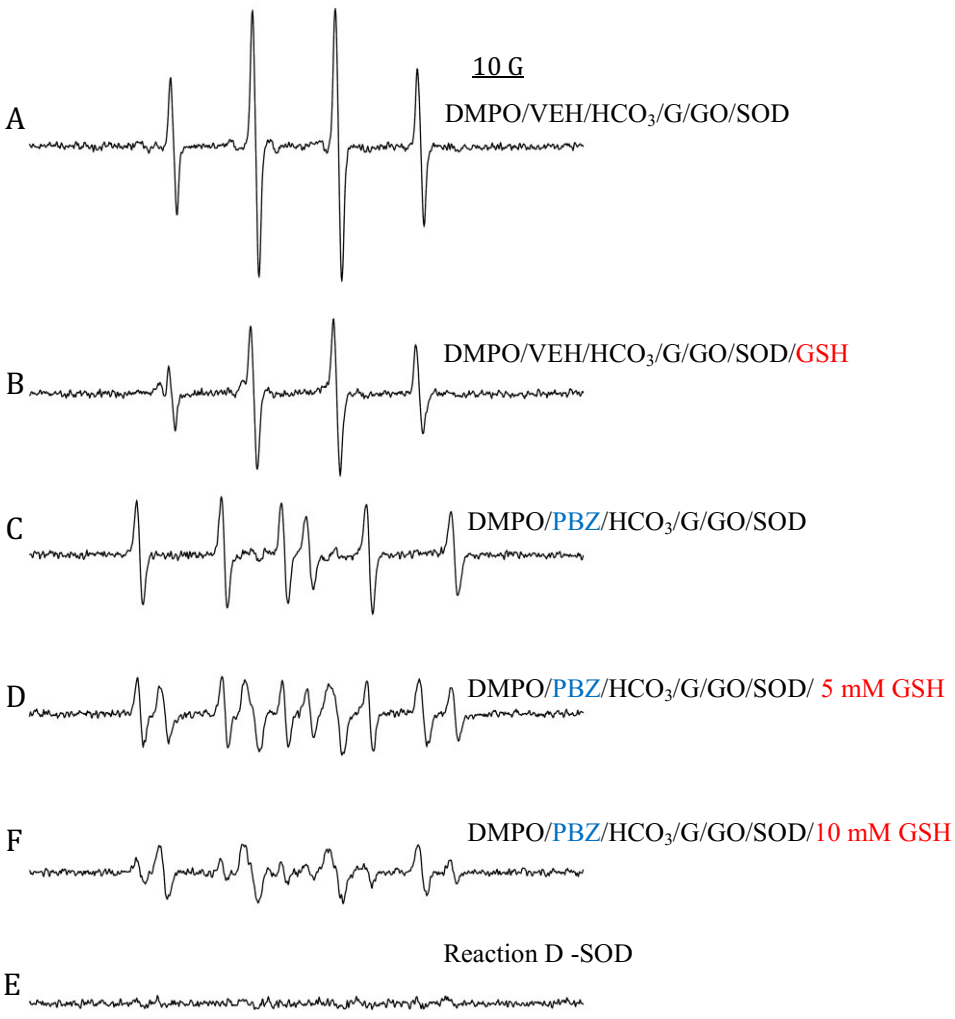

Figure 3.2 Effect of GSH on DMPO/OH and DMPO/PBZ adducts. A spectrum was recorded from a reaction containing 100 mM DMPO, 33 mM HCO_3^-, 25 mM glucose, 7.5 U/200 µL glucose oxidase, and 1 µM bSOD with 0 mM PBZ and 0 mM GSH (A), 0 mMPBZ and 5 mM GSH (B), 1 mM PBZ and 0 mM GSH (C), and 1mM PBZ with different concentrations of GSH, 5mM (D), 10 mM (E). The exclusion of bSOD from reaction (D) showed no free radical detection in the spectrum (F). All reactions were incubated in 0.1 M phosphate buffer containing 100 µM DTPA, pH 7.4 at room temperature for 10 min, and the spectra were immediately recorded after addition of the last reactant (a vehicle or GSH). n=3.

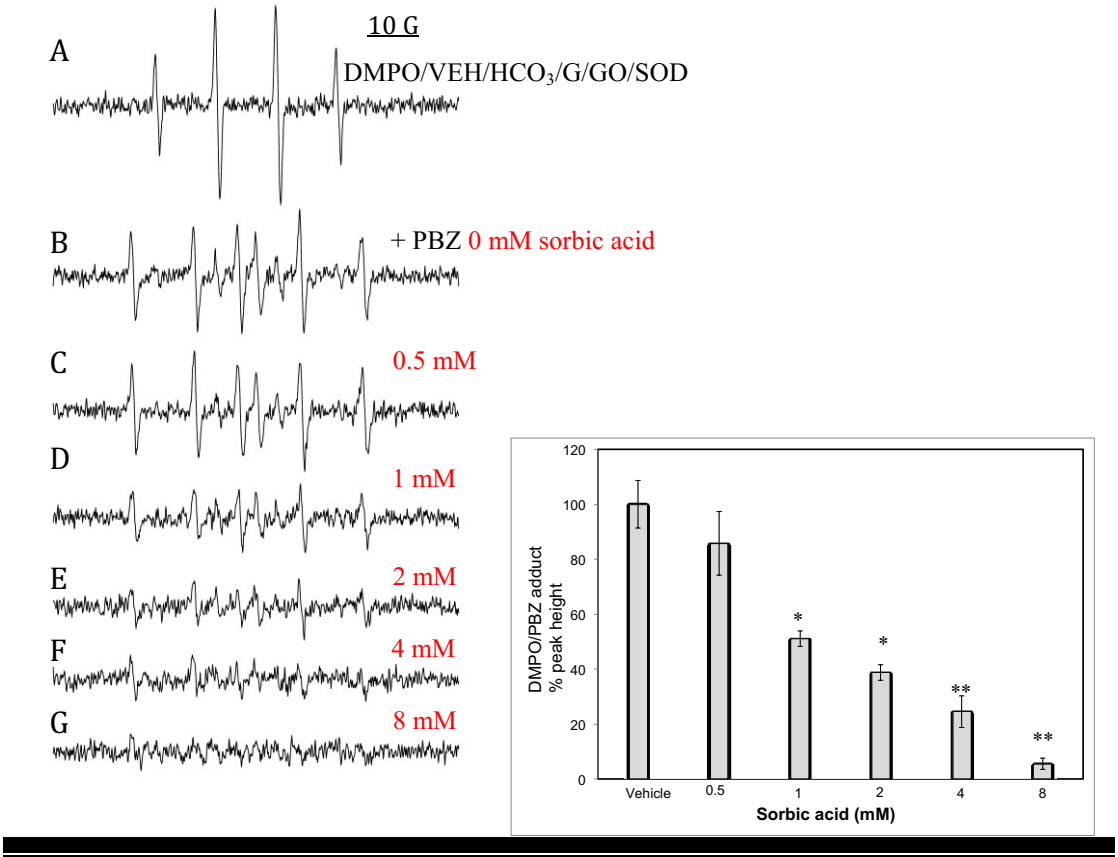

Figure 3.3 Free radical scavenging activity of sorbic acid determined by EPR. Concentration-dependent attenuation of the DMPO•/PBZ adduct with increasing sorbic acid concentration. The spectra were generated by the reaction of 100 mM DMPO, 33 mM HCO_3^-, 25 mM glucose, 7.5 U/200 µL glucose oxidase, and 1 µM bSOD with either 0 µM PBZ and 0 µM sorbic acid (A), or with 1mM PBZ and 0 µM sorbic acid (B), or with 1 mM PBZ and different concentrations of sorbic acid, 0.5 mM (C), 1 mM (D), 2 mM (E), 4 mM (F), 8 mM, (G). All reactions were carried out in 0.1 M phosphate buffer containing 100 µM DTPA, pH 7.4, at room temperature; *, $p < 0.05$, and ** $p < 0.01$ compared with the vehicle and 0.5 mM sorbic acid. n=3.

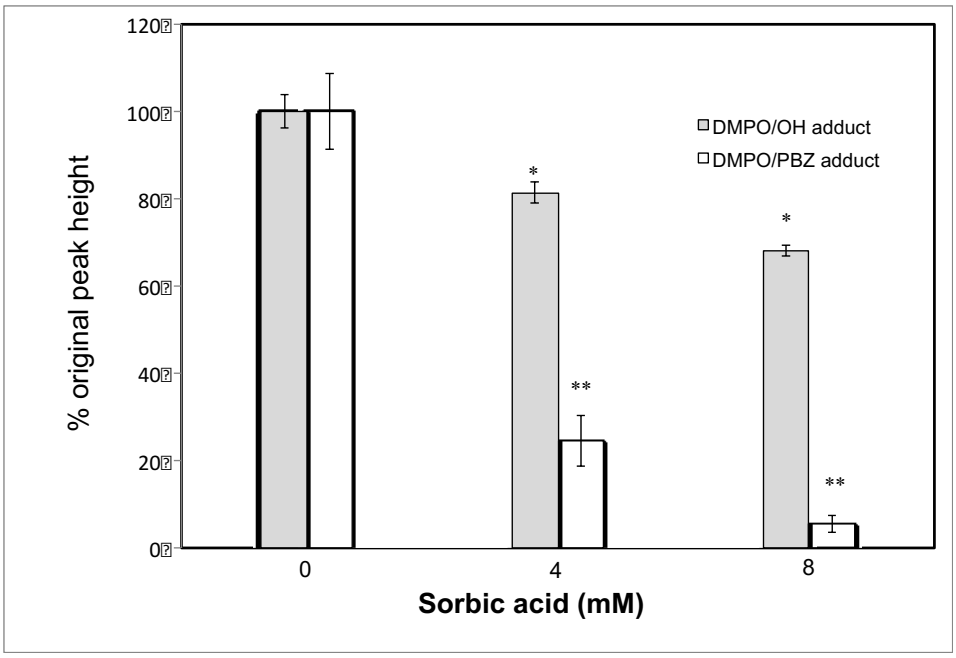

Figure 3.4 Free radical scavenging activity of sorbic acid determined by EPR. The attenuation effect of sorbic acid on DMPO$^{\bullet}$/OH adduct. The signals were generated with the presence of 100 mM DMPO, 33 mM HCO_3^-, 25 mM glucose, 7.5 U/200 μL glucose oxidase, 1 μM bSOD, and different concentrations of sorbic acid 0 mM (A), 4 mM (B), 8 mM (C). All reactions were carried out in 0.1 M phosphate buffer containing 100 μM DTPA, pH 7.4, at room temperature. n=3.

3.4.3 Demonstrating bSOD1 activity in the presence of PBZ, H_2O_2, and HCO_3^-.

The incubation of b SOD1 with H_2O_2 or HCO_3^- resulted in a significant decrease in its dismutase activity; this finding is in agreement with other studies [5,10,13,26]. The deleterious side effects of $CO_3^{\bullet-}$ on bSOD1 activity, per se, were not ameliorated by different concentrations of PBZ; albeit a high concentration of PBZ was used. bSOD1 activity was not changed by the presence of either vehicle or PBZ (Fig. 3.5).

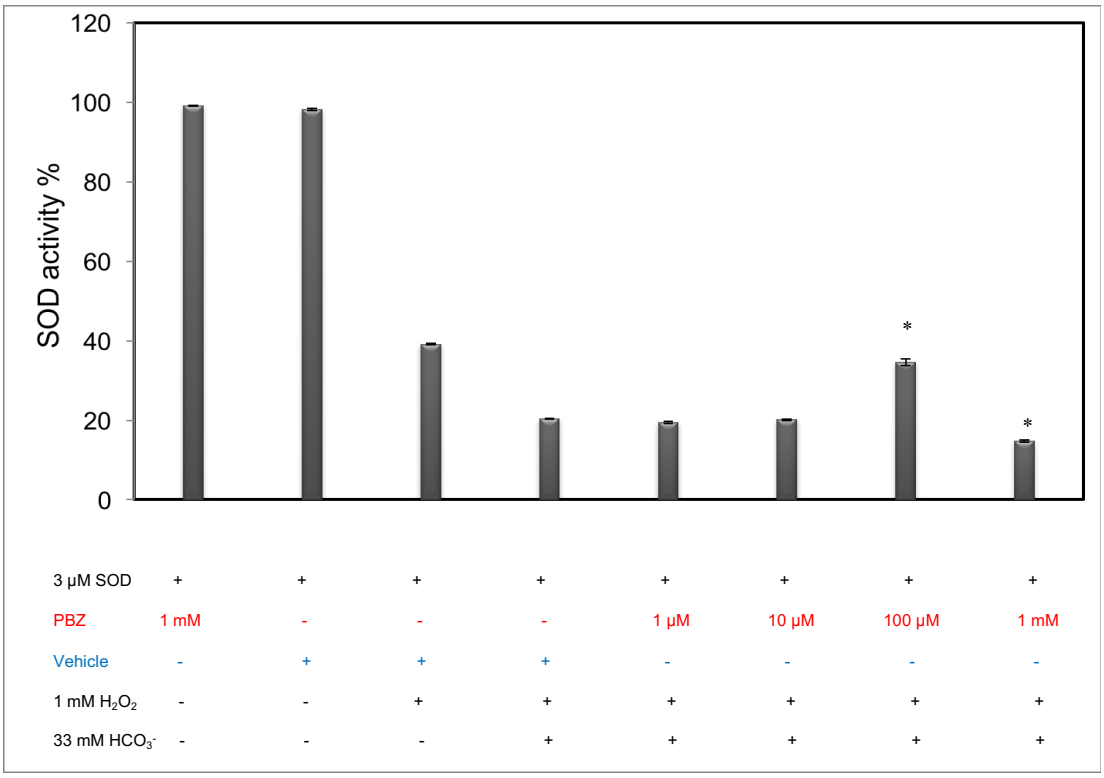

Figure 3.5 Effect of PBZ on oxidative modification of bSOD1. The reaction mixtures contained 3 µM bSOD (2.7 mg/mL), 1 mM H_2O_2, 33 mM HCO_3^-, and either vehicle or different concentrations of PBZ (1 µM, 10 µM, 100 µM , and 1 mM) were incubated for 3 h at 37 ^{O}C. After the reaction was stopped by catalase, bSOD1 dismutase activity was measured as described in the Materials and methods section. Methanol was used as a vehicle. All reactions were carried out in 0.1 M phosphate buffer containing 100 µM DTPA, pH 7.4; *, $p < 0.05$ compared with SOD peroxidase system ($SOD/H_2O_2/HCO_3$). n=3

3.4.4 Effect of sorbic acid and GSH on bSOD1 dismutase inactivation by $CO_3^{\bullet-}$ and PBZ.

In the absence of H_2O_2 and HCO_3^-, SOD dismutase activity did not alter by different concentrations of GSH (data not shown). Different concentrations of sorbic acid could not ameliorate the inactivation of bSOD1 by $CO_3^{\bullet-}$ and PBZ-carbon centered radicals; however, GSH has an ability to scavenge these radicals, leading to significantly restore the dismutase activity of bSOD1 (Figure 3.6). In order to confirm the role of GSH in scavenging PBZ carbon-centered radicals (Figure 3.2) and its ability to suppress the inactivation of bSOD1 by $CO_3^{\bullet-}$ and PBZ-carbon centered radicals, we measured bSOD1 protein through performing SDS/PAGE separation as described in Materials and methods. Densitometric analysis of dimer of b SOD1 showed the density of protein band at 64 kDa was reduced by PBZ combined with H_2O_2 and HCO_3^- (Figure 3.7 Lane 4). However, the b SOD1 band was slightly increased by sorbic acid (0.5 mM and 1mM), suggesting that protein damage by PBZ carbon-centered radical can be slightly attenuated by sorbic acid (Figure 3.7 Lane 6 and 7). Also, b SOD1 damage by $CO_3^{\bullet-}$ and PBZ-carbon centered radicals can be significantly attenuated upon addition of GSH (Figure 3.7 Lane 8 and 9).

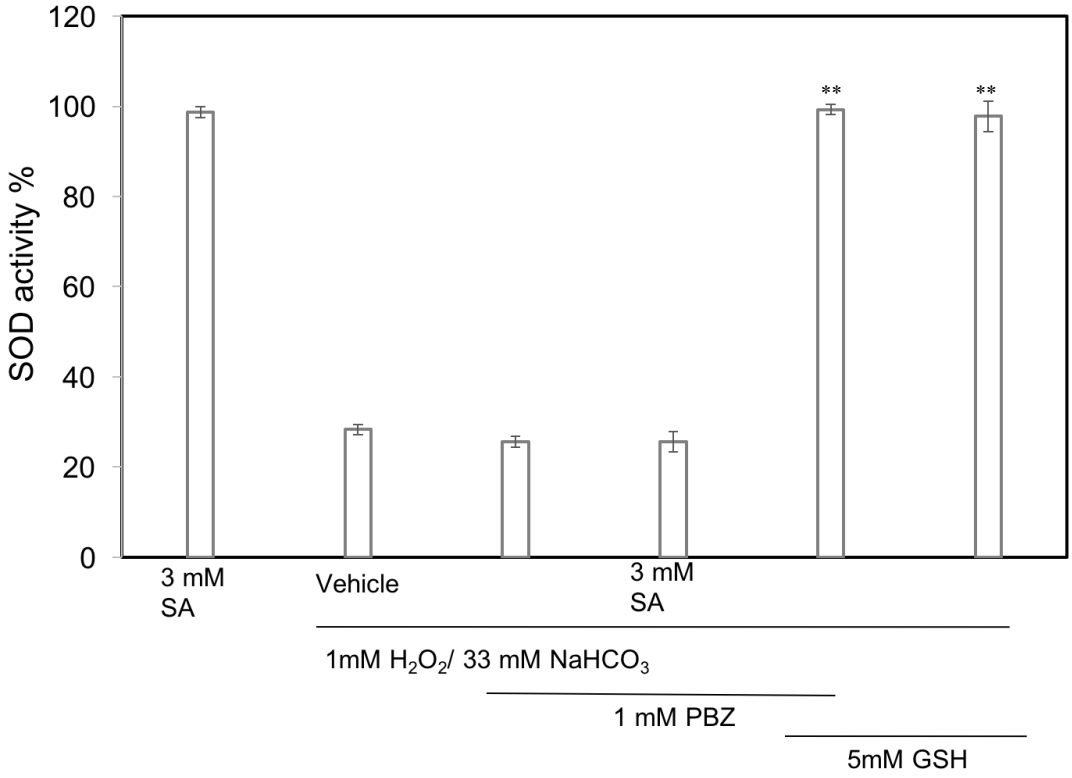

Figure 3.6 Effect of sorbic acid and GSH on the inactivation of bSOD1 by PBZ. bSOD1 dismutase activity measurements expressed as %. 3 μM bSOD (2.7 mg/mL) was incubated with 1 mM H_2O_2, 33 mM HCO_3^-, and either sorbic acid or 5 mM GSH for 3 h at 37 ^OC. All reactions were carried out in 0.1 M phosphate buffer containing 100 μM DTPA, pH 7.4. n=3.

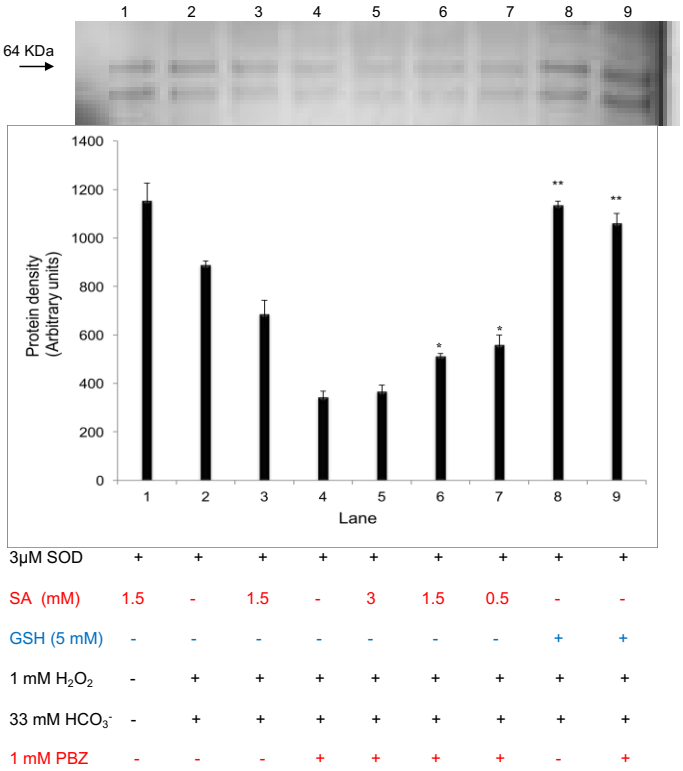

Figure 3.7 Effect of sorbic acid and GSH on bSOD1 protein. SDS/PAGE stained with silver. 3 µM SOD (2.7 mg/Ml) was incubated with mixture containing 33 mM HCO_3, 1 mM H_2O_2, 5 mM GSH, 1 mM PBZ, and either different concentrations of sorbic acid (SA) (0.5, 1.5, and 3 mM) at 37 °C for 3 h. The arrow indicates bands that were quantified by densitometric analysis of dimer of b SOD1. SOD1 separation was performed and all the reactions were carried out in 0.1M phosphate buffer containing 100 µM DTPA, pH 7.4. Values are the means ± SD, ** $P<0.001$, * $P<0.05$ as compared to Lane 4. (n=2).

3.4.5 Effect of combined PBZ and H_2O_2 with the presence or absence DDC on HepG2 cells.

The effect of PBZ/H_2O_2 treatment on HepG2 cells is shown in Figure 3.8. The figure illustrates that the metabolic activity and cell viability of HepG2 cells were significantly decreased in cells treated with the combined treatment of PBZ and H_2O_2 as compared to that treated with either PBZ or H_2O_2 (Fig. 3.8 (c) and 3.8 (d)). These results indicate that combined treatment synergistically enhanced PBZ-induced cytotoxicity in HepG2 cells. We also evaluated the role of SOD1 in PBZ cytotoxicity via using DDC, which is known as an SOD1 inhibitor and a copper chelator (Fig. 3.9 (b)). We found that the cytotoxicity of combined treatment (PBZ and H_2O_2) was significantly attenuated with increasing DDC concentrations (Fig. 3.9(a)), suggesting that SOD1 could potentially catalyze PBZ cytotoxicity in HepG2 cells via peroxidase activity.

To define the mechanism by which PBZ-induced cytotoxicity, we detected the PBZ carbon-centered radicals in HepG2 cells using EPR spectroscopy using DMPO as a spin trap. Both DMPO/$^{\bullet}$OH and DMPO/$^{\bullet}$PBZ spin adducts were detected in intact HepG2 cells (Fig. 3.10 (a) ii and iii); however, the signal intensity of DMPO/$^{\bullet}$OH was not significant if glucose oxidase (H_2O_2 influx system) was omitted from the reaction (Fig. 3.10 (a) iv). In addition, DDC showed a concentration-dependent in a decrease in DMPO/$^{\bullet}$PBZ spin adducts using intact HepG2 cells (Fig. 10 (b) ii-iv). This suggests that PBZ could induce its cytotoxicity in HepG2 cells via forming carbon radicals, a product of oxidizing PBZ by SOD1 peroxidase activity.

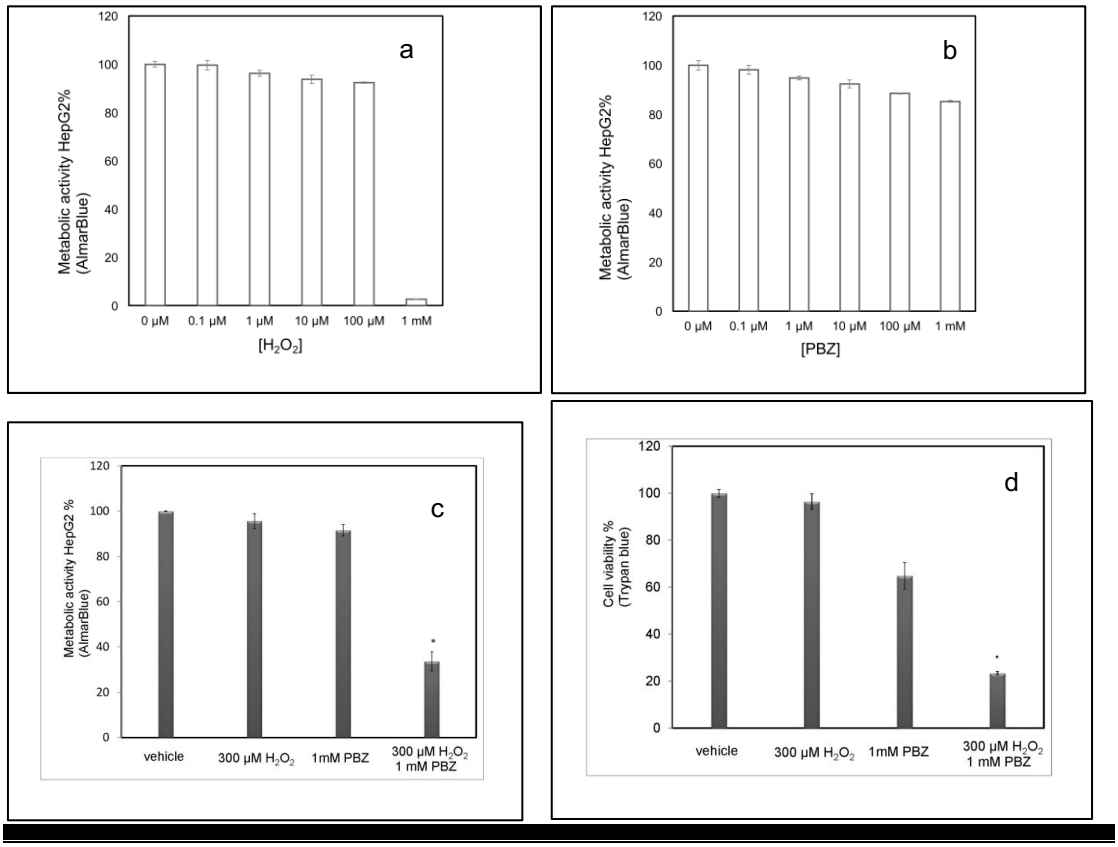

Figure 3.8 Effect of combined PBZ/H_2O_2 on HepG2 cells. The concentration-dependent cytotoxicity of treatment was determined in HepG2 cells through incubating cells with different concentrations of either H_2O_2 (a) or PBZ (b) for 24 h. The effect of combined treatment on the metabolic activity and cell viability of HepG2 was determined by using alamarBlue assay (c) trypan blue (d). HepG2 cells were incubated with either 300 µM H_2O_2 or 1mM PBZ or combined 1mMPBZ/300 µM H_2O_2 for 24 h. Values are the means ± SD, * $P<0.05$ as compared to PBZ (n=5).

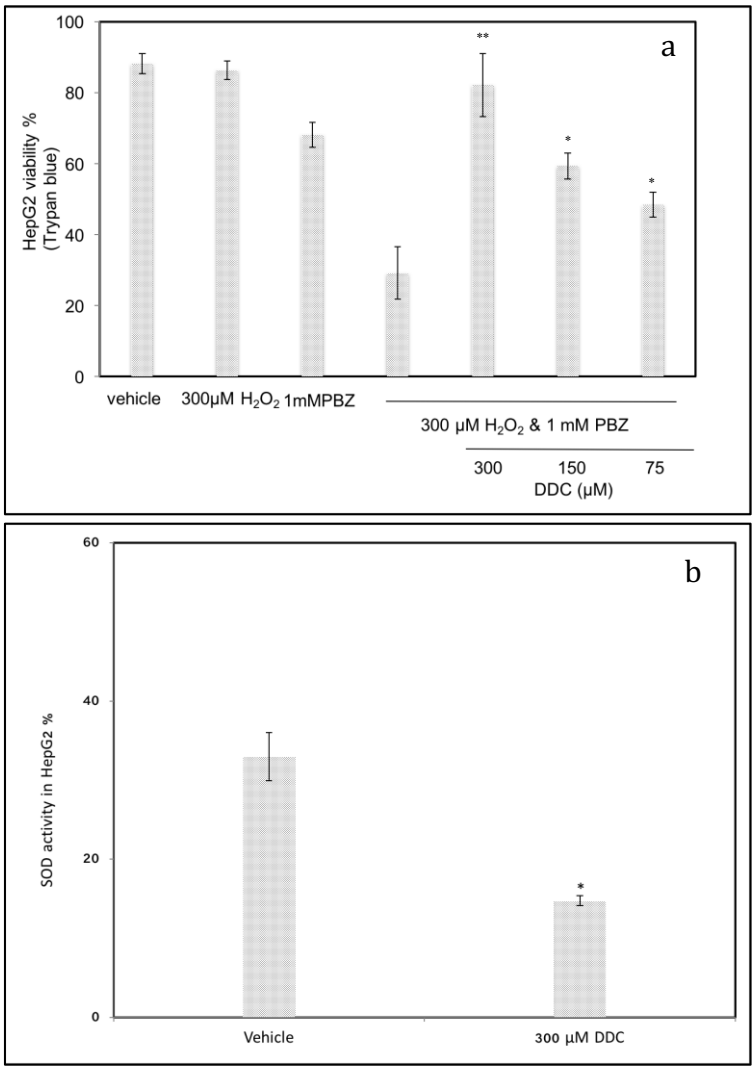

Figure 3.9 Effect of DDC on the cytotoxicity of PBZ/H_2O_2 and SOD activity. HepG2 cells were incubated with combined 1mMPBZ/300 μM H_2O_2 in the absence or presence different concentrations of DDC (75 μM, 150 μM, and 300 μM) for 24 h and the cell viability was determined by using trypan blue assay (a) Values are the means ± SD, ** P<0.001, * P<0.05 as compared to combined PBZ/ H_2O_2 (n=3). In a separate experiment, cells were incubated with either vehicle (deionized H_2O) or 300 μM DDC for 24 h to determine SOD activity (b). Values are the means ± SD, * P<0.05 as compared to vehicle (n=2).

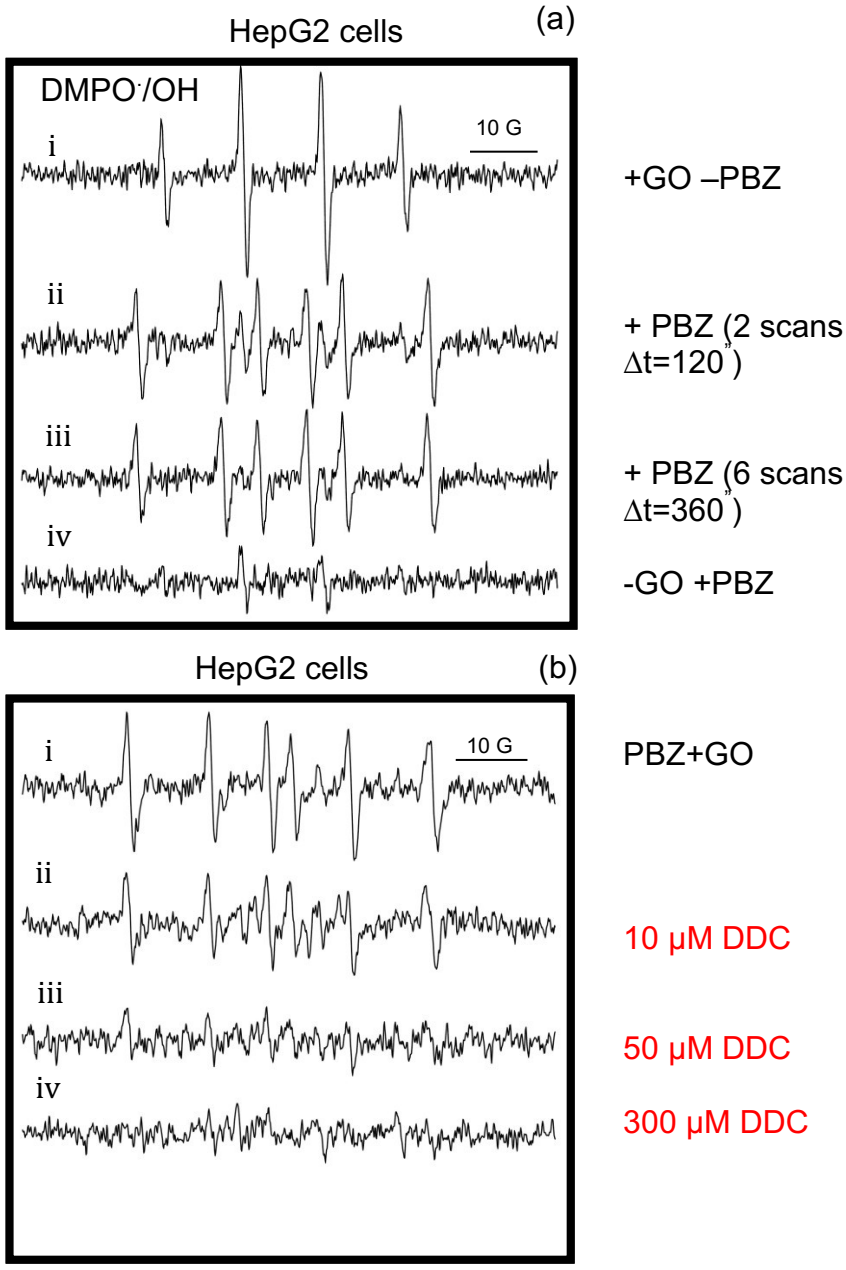

Figure 3.10 Effect of PBZ and DDC on DMPO/PBZ spin adduct formation using intact HepG2 cells. EPR spectroscopy with DMPO spin trap was used to detect both DMPO/•OH and DMPO/•PBZ spin adducts in HepG2 cells (a). The spectrum (a i) generated from incubation of HepG2 cells with 45 mM DMPO, 7.5 U/200 μL glucose oxidase, and vehicle. Both DMPO/•OH and DMPO/•PBZ adducts were detected upon addition of 1mM PBZ to the reaction mixture (a ii; after 2 scans). After 6 scans, DMPO/•PBZ adducts were only detected (a iii). The omission of glucose oxidase from reaction contained HepG2 cells and 1mM PBZ showed no spin

adduct formation (a iv). The DDC caused concentration-dependent attenuation of DMPO/•PBZ adducts (b). The DMPO/•PBZ adducts were formed by incubation HepG2 cells with 45 mM DMPO, 7.5 U/200 µL glucose oxidase, and 1mM PBZ (b i). A 10 µM (b ii), 50 µM (b iii), and 300 µM (b iv) of DDC decreased DMPO/•PBZ adducts. n=2.

3.4.6 Effect of bovine and human SOD1 on PBZ cytotoxicity.

To define the role of SOD1 in PBZ cytotoxicity, we used both exogenous bovine and human SOD1. Interestingly, the cytotoxic effect of PBZ/H_2O_2 on the metabolic activity of HepG2 was significantly enhanced by the presence of both 0.15 µM hSOD1(Figure 3.11) and different concentrations of bSOD1 (A 1.2). These results may suggest that SOD1 could enhance PBZ cytotoxicity in HepG2 via oxidizing PBZ by SOD peroxidase activity.

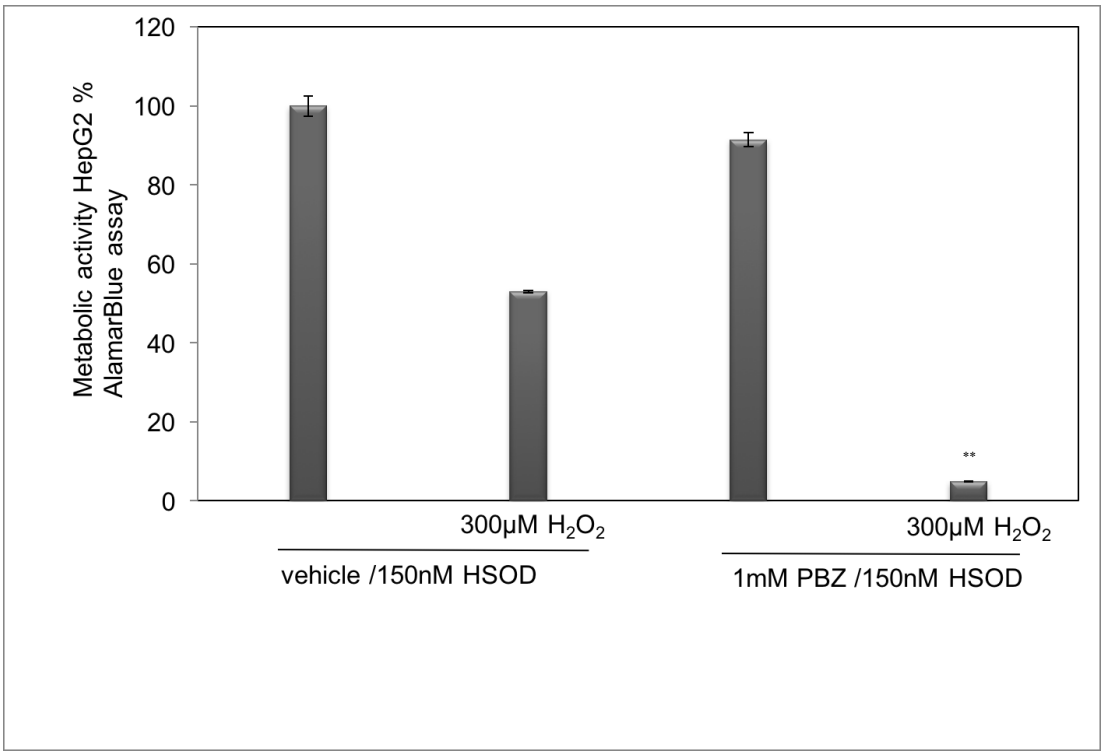

Figure 3.11 Effect of extracellular hSOD1 on the cytotoxic effect of the combined treatment PBZ/H_2O_2. HepG2 cells were treated with combined 1mMPBZ/300 μM H_2O_2 in the absence or presence different concentrations of bSOD1 and incubated for 24 h. The metabolic activity of HepG2 was determined by using alamarBlue assay. ** $P<0.001$ compared to other treatments. (n=2).

3.5 Discussion

In this study, the findings suggest that intra and extra cellular SOD can catalyze PBZ radical formation through SOD peroxidase activity. However, there may other sources of oxidizing PBZ with H_2O_2 a cofactor. Biologically, xanthine oxidase turnover in the presence of HCO_3^-, the homolysis of peroxynitrite carbon dioxide adduct ($ONOOCO_2^-$), the reaction of $HO^\bullet$ with HCO_3^-, and the peroxidase activity of SOD1 could result in forming $CO_3^{\bullet-}$ [1-4]. Although there is an argument about the substrate for peroxidase activity of SOD1 (either $NaHCO_3$ or CO_2), the incubation of SOD1 with both H_2O_2 and HCO_3^- leads to $CO_3^{\bullet-}$ formation [5,6]. Our group showed that SOD1 peroxidase activity could oxidize PBZ by $CO_3^{\bullet-}$, through forming carbon-centered radicals [7]. The reactivity of PBZ carbon-centered radical toward GSH (antioxidant molecules) and sorbic acid (2,4-dihexaenoic acid) is depicted in Figure 3.12.

Copper (Cu^{+2}) and zinc (Zn^{+2}) are in the active site of SOD1 and are necessary for its catalytic activity. It has been reported that Cu^{+2}-SOD1 can be reduced by $O_2^{\bullet-}$, yielding to Cu^{+1}-SOD1 that could be also oxidized by another molecule of $O_2^{\bullet-}$, culminating in reproducing Cu^{+2}-SOD1 with H_2O_2 and O_2 formation, this is called a dismutase activity [8,9]. However, H_2O_2, per se, could oxidize Cu^{+1}-SOD1, leading to a strong oxidant molecule (SOD-OH) that could oxidize a number of substrates, resulting in oxidative stress. In particular, SOD-OH oxidation the ubiquitous bicarbonate anion results in $CO_3^{\bullet-}$ formation [5,10,11].

In order to detect a free radical with an unpaired electron, we used EPR spectroscopy technique, which is the most unambiguous technique in free radical detection. In this study, we used EPR with the spin trap DMPO to stabilize and consequently measure

short-lived free radicals, namely $CO_3^{\bullet-}$, PBZ carbon-centered radical, and thiyl radical ($GS^{\bullet}$) [12]. There is a growing consensus of considering DMPO/$^{\bullet}$OH as an indirect evidence for trapping $CO_3^{\bullet-}$, which cannot be directly detected via forming a stable spin adduct with DMPO as compared with other free radicals [4,7,12,13,14]. By using this technique, we found that DDC, an SOD1 inhibitor, concentration-dependently decreased DMPO/$^{\bullet}$OH adduct formation from SOD1 peroxidase activity (Fig. 3.1). It is known that DDC forms a stable complex with Mn^{+3}, Fe^{+3}, Co^{+3}, Ni^{+2}, Cu^{+2}, Zn^{+2}, Hg^{+2}, Pb^{+2}, and Bi^{+3} [15]. This may suggest that the ability of DDC to chelate the active site of SOD1 could result in inhibiting SOD1 dismutase activity. Our group previously showed that DDC and TPEN, zinc chelator, were capable of inhibiting SOD1 in a murine hepatoma cell line, Hepa1c1c7 [16]. Thus, chelating the Cu^{+2} in the active site of SOD1 could lead to decrease both the dismutase and peroxidase activities of SOD1 through repressing $O_2^{\bullet-}$ neutralization and SOD-OH formation, respectively.

In order to investigate the role of PBZ-SOD1 peroxidase activity in oxidizing antioxidant molecules, namely GSH, we carried out further experiments by using EPR. It is pertinent to point out that normal cells contain a high level of GSH, which acts as a major anti-oxidant in protecting cells against ROS and oxidative stress, and is most concentrated in the liver (up to 10 mM) [17]. A number of thiol compounds including GSH could be oxidized by peroxidases in the presence of H_2O_2, leading to thiyl radicals via either hydrogen abstraction or cleavage of disulfide linkage [18]. However, oxidizing GSH by PBZ-HRP-H_2O_2 system showed only diminished in the intensity of PBZ radicals without detecting thiyl radicals [19]. Interestingly, we found that GSH decreased DMPO/$^{\bullet}$PBZ adduct formation with the increase in thiyl

radicals' formation (DMPO/$^{\bullet}$SG) (Fig. 3.2). Dociu et al. demonstrated that PBZ treatment could cause a significant depletion of GSH with an increase in the level of MDA in the liver and renal cortex of female rats [20]. Taken together that oxidizing PBZ by SOD1 peroxidase activity and the ability of GSH to scavenge PBZ radicals via forming thiyl radical could result in GSH depletion.

It is interesting to note that sorbic acid, an antimicrobial agent, is easily attacked by nucleophiles, such as sulfite and amine, as it contains a conjugated double bond in its structure [21]. Interestingly, we found that sorbic acid showed a concentration-dependent attenuation of DMPO/$^{\bullet}$PBZ; with the slight decrease in the intensity of $CO_3^{\bullet-}$ radicals (Fig. 3.4). In the reaction of aniline derivatives with HRP/H_2O_2 system, our group showed that the oxygen consumption was significantly increased in the presence of sorbic acid, as compared to linoleic acid, a polyunsaturated omega-6 fatty acid [22]. This may suggest that sorbic acid could be susceptible to attack by PBZ carbon-centered radical and subsequently could culminate in forming sorbic acid carbon-centered radical, albeit we did not detect this radical even at high sorbic acid concentrations. Nevertheless, low concentrations of sorbic acid apparently scavenged PBZ carbon centered radicals via addition across double bond and potentially could ameliorate the deleterious side effects of PBZ.

Another consequence of PBZ radicals was damage to SOD protein. The consensus is that proteins aggregation and fragmentation could result from irreversible oxidation of protein residues, including cysteine, methionine, tryptophan, histidine, and phenylalanine, which play a role in causing dysfunction and subsequently various human pathologies [23]. The $CO_3^{\bullet-}$ could oxidize a number of amino acids, culminating in protein aggregation, including SOD1, per se, [24,25]. Since our group

showed that PBZ could scavenge $CO_3^{\bullet-}$ radicals, it was important to determine the effect of scavenging these radicals by PBZ on SOD1 activity [7]. It has been reported that WST-8 formazan can be oxidized by 20 mM H_2O_2 in the presence of 37% HCl in methanol at 25 °C for overnight [44], suggesting that oxidizing WST-8 formazan to WST-8 required a high concentration of H_2O_2 and incubation under specific conditions for a long time. However, we found that the incubation of xanthine/xanthine oxidase "enzyme working solution" with WST-1 "WST working solution" led to WST-1 formazan formation that was not oxidized back to WST-1 by 3 mM H_2O_2 (data not shown). In agreement with other studies, our finding showed that the presence of bicarbonate and H_2O_2 significantly decreased SOD1 activity (Fig. 3.5) [6,8,26]. Intriguingly, H_2O_2, per se, can cause a dissociation of a dimer of b SOD1to monomer that, in part, is in agreement with the study that showed b SOD1 concentration, temperature, and urea could be contributed to the dissociation of b SOD1 [45]. However, this deleterious effect of $CO_3^{\bullet-}$ on SOD1 activity was not ameliorated by different concentrations of PBZ. It is noteworthy that α -hydroxyl-containing carbon-centered radicals could be scavenged by tryptophan, 5-hydroxytryptophan [27], suggesting that oxidizing PBZ-carbon centered radicals could potentially oxidize SOD1 residues and subsequently suppress its dismutase activity via oxidizing amino acids residues including tryptophan, as evidenced by protein quantity (Fig.3.7). Interestingly, GSH showed a significant protection against both $CO_3^{\bullet-}$ and PBZ-carbon centered radical that subsequently protected SOD1 (Fig. 3.7) and its dismutase activity (Fig. 3.6). This may indicate that forming thiyl radicals a product of oxidizing PBZ-carbon centered radical (Fig. 3.2) could rapidly convert to GSH disulfide via recombination [28]. By taking together that forming

PBZ-carbon centered radicals by SOD1 peroxidase activity as well as the ability of carbon-centered radicals, per se, to oxidize tryptophan residues, PBZ could potentially suppress the activity of SOD1 through oxidizing SOD1 tryptophan residue.

We performed further studies to investigate the effect of SOD1 peroxidase system on PBZ cytotoxicity via utilizing HepG2 cells. It has been shown that HepG2 cells could produce and secrete SOD1 [29], and the catalase activity in HepG2 cells is low [30], suggesting that there is potential for accumulation H_2O_2. It has been reported that a high concentration of H_2O_2 could inactivate antioxidant enzymes including catalase and glutathione peroxidase 1 [6,31,32]. Interestingly, our experiments showed that combination of H_2O_2 and PBZ resulted in significantly decreased both metabolic activity and cell viability of HepG2 cells (Fig. 3.8 (c) and (d)). To confirm the role of SOD1, we used DDC, a strong copper chelator and SOD1 inhibitor, and we found that the effects of PBZ/H_2O_2 on both metabolic activity (data not shown) and cell viability of HepG2 cells were attenuated (Fig. 3.9 (a)). Although our findings showed both DMPO/$^{\bullet}$PBZ and DMPO/$^{\bullet}$OH adduct formation upon addition PBZ and G/GO via utilizing intact HepG2 cells and these adducts were attenuated by increasing the concentration of DDC, the main source of causing these radicals is still elusive as HepG2 cells contain different metalloenzymes and peroxidase enzymes (Fig. 3.10 (a) and (b). Using G/GO system is mainly to generate H_2O_2 and to repress the reaction of O_2 molecules with PBZ-carbon centered radicals as our group previously showed that PBZ carbon-centered radical reacted with O_2 that culminated in the formation of both 4-hydroperoxyPBZ and 4-hydroxyPBZ [7].

In summary, PBZ-carbon centered radicals, a product of SOD1 peroxidase activity, were decreased by DDC, GSH, and sorbic acid. Both sorbic acid and PBZ did not ameliorate bSOD1 dismutase activity; GSH appeared to scavenge both $CO_3^{\cdot -}$ and PBZ carbon-centered radicals and subsequently protected bSOD1 against oxidant radicals. There was a synergistic cytotoxic effect of combination H_2O_2 and PBZ on HepG2 cells; however, DDC attenuated these effects via decreased PBZ-carbon centered radicals produced in HepG2. Extracellular bSOD1 peroxidase activity enhances the cytotoxic activity of PBZ. Overall, these studies further support the view that the combination of PBZ with SOD1 peroxidase system can culminate in forming PBZ-carbon centered radicals that are relatively unstable and highly reactive towards proteins. Future studies should distinguish the catalytic potency of bSOD vs hSOD peroxidase systems as well as the role of these peroxidases in the molecular mechanism of PBZ induced-toxicity.

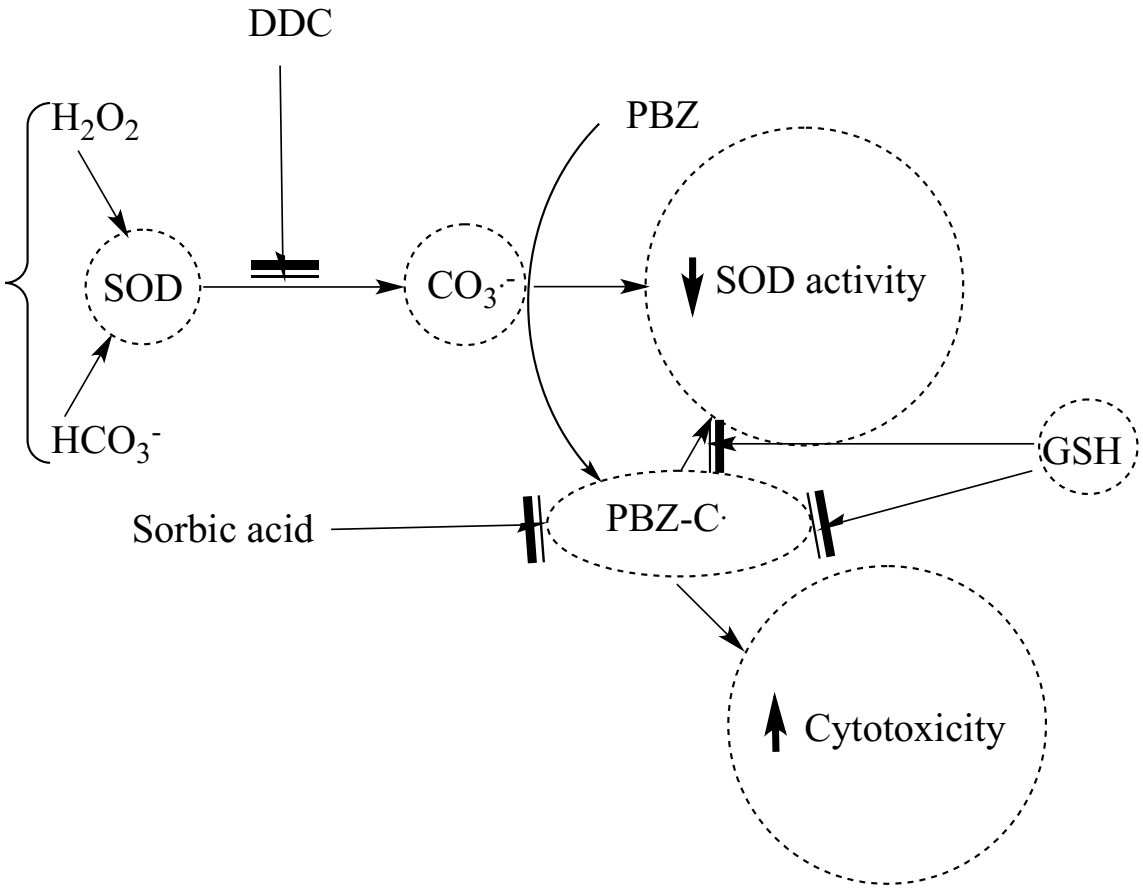

Figure 3.12 Proposed mechanistic pathway of the reactivity of PBZ carbon-centered radicals. The carbonate radical produced from SOD peroxidase activity repressed with DDC, consequently decreased a C-centered radical formation. The C-centered radical scavenged by antioxidant molecules, GSH and conjugated double bond, sorbic acid.

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Chapter 4

Role of Cu-Zn-SOD peroxidase activity in oxidizing 6-mercaptopurine (6-thiopurine derivatives)

"Potential involvement of SOD and peroxymonocarbonate in the oxidation of anticancer drug metabolite, 6-mercaptopurine"

…….

4.1 Abstract

Azathioprine and its metabolite (6-MP) are mainly used in the treatment of inflammatory bowel disease. At sites of inflammation, reactive oxygen species (ROS) are generated, including hydrogen peroxide (H_2O_2). The latter product is known to inactivate superoxide dismutase (SOD), which enhances oxidative stress. H_2O_2 can also catalyze SOD peroxidase activity that can produce carbonate radical ($CO_3^{\cdot-}$) through oxidation of bicarbonate (HCO_3^-). H_2O_2 and HCO_3^- can form peroxymonocarbonate (HCO_4^-). Both $CO_3^{\cdot-}$ and HCO_4^- are known to oxidize electron-rich species such as thiols. 6-MP has a thiol moiety in its structure, but it is not known if SOD peroxidase-derived $CO_3^{\cdot-}$ or HCO_4^- can react with this drug. UV-Vis spectra showed that both bicarbonate-activated peroxide (potential HCO_4^-) and SOD peroxidase-derived $CO_3^{\cdot-}$ induced the most prominent changes with 6-MP and 6-thioguanine; less spectral changes were observed with 6-thioxanthine and 6-thiouric acid. Kinetic studies demonstrated that the changes in the 6-MP peaks were dependent on HCO_3^-/H_2O_2 concentrations. The oxidation of 6-MP by either HCO_3^-/H_2O_2 or SOD peroxidase activity resulted in forming a major peak product ($C_5H_4N_4O_2S$; 182.9981 m/z), which corresponds to 6-sulfoxide-MP, as judged by using high-resolution LC/MS. Using EPR spectroscopy and 5,5-dimethyl-1-pyrroline-N-oxide (DMPO) as a spin trap, we found that the spin adduct of $CO_3^{\bullet-}$ (DMPO/$^{\bullet}$OH) was attenuated with increasing of 6-MP concentrations. However,

DMPO/$^{\bullet}$OH was not significantly attenuated with the presence of azathioprine. Interestingly, inactivation of SOD by H_2O_2/HCO_3^- was significantly attenuated with the presence of 6-MP in a concentration-dependent manner, further indicating that SOD peroxidase-derived $CO_3^{\bullet-}$ reacted with 6-MP. Lastly, pre-incubation of 6-MP with H_2O_2/HCO_3 resulted in significantly decreasing in its cytotoxic effect on both HepG2 and HEK293 cells.

Exposure of 6-MP to both H_2O_2 /HCO_3^- and SOD peroxidase-generated $CO_3^{\bullet-}$ culminated in forming 6-sulfoxide-MP that attenuated 6-MP cytotoxicity of the drug and consequently prevented SOD inactivation.

4.2 Introduction

Azathioprine (AZA) and its metabolite, 6-mercaptopurine (6-MP) are used in the treatment of a variety of diseases, including inflammatory bowel diseases (IBD) and cancers [31]. 6-MP was initially evaluated as an anti-leukemic drug, and then AZA with its metabolite 6-MP was introduced as an immunosuppressant for organ transplantation. After further clinical observations of thiopurine therapy in IBD, it was found that thiopurines are effective in the treatment of both ulcerative colitis and Crohn's disease [31]. 6-MP is formed by cleavage of AZA (prodrug) *in vivo* non-enzymatically by glutathione and undergoes a series of enzymatic reactions that culminate in forming its active metabolite, 6-thioguanine nucleotide (6-TGN); the latter is the precursor for the incorporation of 6-thioguanine into RNA and DNA. The addition of ribose 5-phosphate to 6-MP and 6-TG catalyzed by hypoxanthine-guanine phosphoribosyltransferase (HPRT), leads to generate 6-thioinosine

monophosphate and 6-thioguanosine monophosphate, respectively; this process is the first step in forming 6-TGN that subsequently blocks the formation of purine nucleotides and inhibits DNA synthesis, this is known as mismatch repair pathway [32]. However, thiopurine methyl transferase (TPMT), xanthine oxidase and aldehyde dehydrogenase can catalytically inactivate 6-MP, yielding 6-methylmercaptopurine, 6-thioxanthine (6-TX) and 6-thiouric acid (6-TUA), respectively (Fig. 4.1) [33].

It has been shown that AZA and its metabolite (6-MP) exert their effects in treating IBD through formation 6-TGN, which incorporates into cellular nucleic acids, that culminating in inhibition of nucleotide and protein synthesis and consequently inhibition of lymphocyte proliferation [34,35]. However, there may be other mechanisms by which 6-thiopurine drugs exert their effects. It is believed that sites of inflammation sites are characterized by the generation of reactive oxygen species (ROS) that have an ability to oxidize thiol-containing compounds. The oxidation of thiol compounds plays a pivotal role in a variety of biological processes, including the response of antioxidant. It has been reported that levels of superoxide dismutase (SOD) protein and activity in peripheral blood granulocytes of IBD patients are low, and also the activities of both catalase and GSH peroxidase1 are suppressed in Crohn's patients [36], suggesting that accumulation of ROS could culminate in generating the oxidative stress.

SODs are classified based on their locations: SOD 3 (Cu-Zn SOD) is located in the extracellular space; SOD 1 (Cu-Zn SOD) and SOD 2 (Mn SOD) are located in the cytoplasm and mitochondria, respectively [8]. The main function for SODs is to

eliminate the superoxide anion radicals ($O_2^{\bullet-}$) that are normally produced inside the cells, yielding hydrogen peroxide (H_2O_2); this is known as dismutase activity [1-4]. However, SODs are inactivated with the potential accumulation of H_2O_2, culminating in the formation of a localized hydroxyl radical (SOD-OH); this is a peroxidase activity of SOD [7,19,21].

The SOD-OH can oxidize bicarbonate, resulting in $CO_3^{\bullet-}$ radical formation; this is known as bicarbonate-dependent peroxidase activity of SOD [19,20]. The $CO_3^{\bullet-}$ radical (E^o $CO_3^{\bullet-}$/ HCO_3^-=1.8 V) has an ability to diffuse and oxidize various biological targets, including hSOD1 [19,26]. Both electron transfer and hydrogen abstraction are the main mechanisms by which $CO_3^{\bullet-}$ oxidizes targets. For example, human-SOD (hSOD1) aggregation results from oxidizing tryptophan residues by $CO_3^{\bullet-}$, leading to non-native hSOD1; this is mainly attributed to the pathogenesis of familial amyotrophic lateral sclerosis (ALS), a motor neuron disease [37]. Rats that overexpressed mutant $hSOD1^{G93A}$ have moderately increased survival if administered high doses of tempol (4-hydroxy-2,2,6,6-tetramethyl piperidine-1-oxyl), an antioxidant and cyclic nitroxide [38]. Nevertheless, there may yet be other drugs that have an ability to scavenge $CO_3^{\bullet-}$ radical.

Peroxymonocarbonate (HCO_4^-) is a product of the reaction of H_2O_2 with either CO_2 or HCO_3^- that catalyzes the oxidation of various molecules including tertiary amines, organic sulfides and thiols [10-12]. Also, HCO_4^- is capable of oxidizing sulfur-containing biomolecules, such as methionine and cysteine [12]. Although the rate constant of the formation of HCO_4^- is relatively slow 2.4 X 10^{-3} $M^{-1}S^{-1}$ (H_2O_2 reacting with HCO_3), the HCO_4^-, per se, is unstable and easily hydrolyzed or reduced to form H_2O_2/HCO_3^- and $CO_3^{\bullet-}$ radicals [11,12,15,16].

At sites of inflammation, the accumulation of ROS including H_2O_2 is likely, that is known to inactivate SOD, culminates in forming $CO_3^{\bullet-}$ by oxidizing HCO_3^-. Since 6-MP and AZA are used in treating IBD, it is plausible that these drugs could interact with ROS. Therefore, we hypothesized that SOD peroxidase-derived $CO_3^{\bullet-}$ would catalyze the oxidation of 6-MP by bicarbonate-activated peroxide system (potential HCO_4^-).

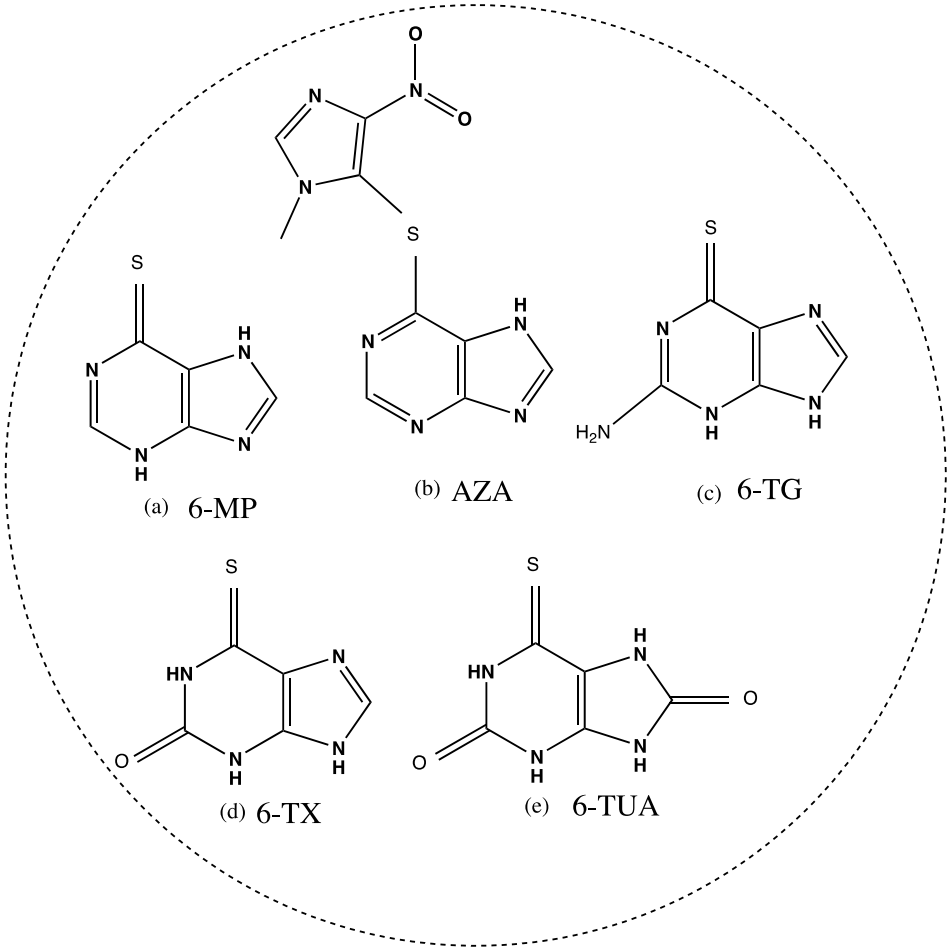


Figure 4.1 Chemical structures of thiopurine derivatives. (a) 6-MP, (b) AZA, (c) 6-TG, (d) 6-TX, (e) 6-TUA.

4.3 Experimental procedures

4.3.1 Reagents and kits

6-MP, sodium phosphate monobasic, sodium phosphate dibasic, sodium hydroxide, potassium chloride, magnesium sulfate, hydrogen peroxide (H_2O_2), $NaHCO_3$, diethylene triamine pentaacetic acid (DTPA), Dulbecco's phosphate buffer saline (DPBS), and 6-TG were purchased from Sigma Aldrich Canada Co. (Oakville, ON). 5,5-Dimethyl-1-pyrroline-N-oxide (DMPO) was purchased from Cedarlane Laboratories and was determined to be of high purity (such that vacuum distillation was not required). SOD Assay Kit-WST was purchased from Dojindo Molecular Technologies, Inc., (Maryland, USA) via Cedarlane Laboratories Ltd. Azathioprine, 6-thioxanthine (6-TX) and 6-thiouric acid (6-TUA) were purchased from Toronto Research Chemicals (Toronto, ON). Bovine SOD was purchased from MP Biomedical, LLC (Santa Ana, CA). All solutions and buffers were prepared with deionized water. The H_2O_2 solution was prepared just before use in all experiments. The pH measurements were recorded on a Fisher Scientific Accumet^R pH meter (Waltham, MA).

4.3.2 Spectrophotometric analysis

UV-Vis absorption scans were performed on a Spectra Max M5 Multi-Mode Microplate Reader (Molecular Devices, Sunnyvale CA). Optical spectra were recorded by utilizing a 1 cm path length quartz cuvette. The reactions contained 25 mM HCO_3^-, 1 mM H_2O_2 and with the presence of either 20 μM AZA or 20 μM of its metabolites. As the most prominent changes were observed with 6-MP, the kinetic

measurement modes at 319 nm and 272 nm were carried out in the presence of different concentration of H_2O_2 (250 μM, 500 μM, and 1mM) and 25 mM HCO_3^-; the kinetic spectra were recorded for 6 min. using a Thermo Scientific NanoDrop 2000c (Wilmington, DE). All the reactions were carried out in 0.1 M phosphate buffer containing 100 μM DTPA, pH 7.4, at room temperature.

4.3.3 Mass spectrometry analysis

RP-HPLC- MS was performed using an Agilent 1200 SL HPLC System with an Altima HP HILIC column, 2.1x50mm with guard, 3 μm particle size, thermostated at 30°C, with a buffer gradient system composed of acetonitrile (ACN) as mobile phase A and 20 mM ammonium formate in water, pH4.4, as mobile phase B. An aliquot of 2 μL was loaded onto the column at a flow rate of 0.3 mL min^{-1} using an initial buffer composition of 98% mobile phase A and 2% mobile phase B. After injection, the column was kept at the initial loading conditions for 2.0 minutes followed by elution of the analytes by using a linear gradient from 2% to 30% mobile phase B over a period of 5 minutes, 30% to 65% mobile phase B over 3 minutes, 65% to 85% B over 2 minutes, held at 85% mobile phase B for 1.9 minutes to remove all analytes from the column. Mass spectra were acquired in negative mode ionization using an Agilent 6220 Accurate-Mass TOF HPLC/MS system (Santa Clara, CA, USA) equipped with a dual sprayer electrospray ionization source with the second sprayer providing a reference mass solution. Mass spectrometric conditions were drying gas 9 L/min at 300°C, nebulizer 20 psi, mass range 100-1100 Da, acquisition rate of ~1.03 spectra/sec, fragmentor 140 V, skimmer 65 V, capillary 3200 V, instrument

state 4GHz High Resolution. A mass correction was performed for every individual spectrum using peaks at m/z 112.98558 and 1033.98811 from the reference solution. Data acquisition was performed using the Mass Hunter software package (ver. B.04.00.). Analysis of the HPLC-UV-MS data was done using the Agilent Mass Hunter Qualitative Analysis software (ver. B.07.00).

4.3.4 Electron paramagnetic resonance (EPR) spin trapping measurements. EPR spin trapping experiments were performed using a Bruker Elexys E-500 spectrometer (Billerica, MA) with the following instrument parameters: frequency: 9.81 GHZ, center field: 3497 G, microwave power: 20 mW, modulation amplitude: 1.0 G, modulation frequency: 100 kHz, sweep time: 60 s, scan number: 2. After measuring the pH, 200 µL of the reaction was transferred to a suprasil quartz flat cell and the scan was started immediately. The DMPO/$^{\bullet}$OH, which is indirect evidence for $CO_3^{\bullet-}$, was detected directly in the presence of 1 µM SOD, 33 mM HCO_3, 1 mM H_2O_2 and also with the presence of different concentrations of 6-MP (50 µM, 100 µM, 200 µM and 400 µM). Similar experiments were performed to compare the effect of azathioprine on DMPO/$^{\bullet}$OH formation upon addition of 400 µM azathioprine. All reactions were performed in 0.1 M phosphate buffer pH 7.4 containing 100 µM DTPA, at room temperature.

4.3.5 SOD protein and activity assay

The measurements of SOD protein and activity were performed as previously described with slight modifications [26]. 3 µM SOD (2.7 mg/mL) was incubated

with the mixture containing 33 mM HCO_3^-, 1 mM H_2O_2, and either different concentrations of 6-MP (200, 350, 450, 600, and 750 μM) or 750 μM azathioprine at 37 °C for 6 h. The SOD dismutase activity was measured by using SOD-WST Kit according to manufacturer's instructions.

After incubation for 6 h at 37 °C, the protein was precipitated by addition of ice-cold acetone (1 mL for each reaction), all the reaction mixtures were kept at -20 °C for 4 h and then were centrifuged at 16000 g for 15 min. The pellets were washed three times with Dulbecco's phosphate buffer saline, and the concentration of protein was determined by using BCA protein assay (Biorad laboratories, Mississauga, ON). 10 μg of protein aliquots were separated via using SDS-PAGE (15 % gel). After washing with TBS, the gels were fixed with methanol (50 %) and acetic acid (10 %) for 10 min. We used Image J to relatively quantify of the bands. The gels were stained with Coomassie Blue for 5 h. at room temperature.

4.3.6 Cell culture

The human hepatocellular carcinoma cells (HepG2) and human embryonic kidney 293 cells (HEK293) were obtained from ATCC, and both cells were maintained in Dulbecco's modified Eagle's medium (DMEM; Invitrogen), supplemented with 10% fetal bovine serum (Thermo Scientific), 1% penicillin-streptomycin, 1% non-essential amino acids. Cells were grown in 75-cm^2 cell culture flasks at 37 °C in a 5% CO_2 humidified atmosphere for 72 h. After washing the cells with DPBS, cells were detached by trypsinization and followed by centrifugation at 300g at 4 °C for 5

min. Cells were seeded at a density of 10 x 10^3 in 96-well and incubated at 37 °C in humidified incubator, 5% CO_2, for 24 h, prior to the treatments.

4.3.7 HepG2 and HEK293 cells treatments

Initially, 6-MP was pretreated with or without H_2O_2 and HCO_3^- before adding to cells. Different concentrations of 6-MP (1, 5, 10, 100 μM), or 25 μM H_2O_2 alone, or H_2O_2 combined with different concentrations of 6-MP were incubated in 0.1M phosphate buffer containing, 25 mM HCO_3 and 100 μM DTPA, pH 7.4 for 60 min before adding to cells. Both HepG2 and HEK293 cells were incubated at 37 °C in humidified incubator, 5% CO_2, for 72 h, after treatment with 6-MP or H_2O_2, and the metabolic activity of both cells were determined by using MTT assay. The metabolic activity in both cells was measured based on converting 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) to formazan crystals via reducing enzymes present in viable cells as described previously [39].

4.3.8 Statistical analysis

Results are expressed as the mean ± SD. For EPR studies, representative spectra were shown. The differences were evaluated by one-way ANOVA or Student's t-test, followed by Student-Newman-Keuls post-hoc test. Values with $^{*}P < 0.05$, $^{**}P < 0.01$ were considered statistically significant.

4.4 Results

4.4.1 Bicarbonate catalyzed H_2O_2-oxidized 6-thiopurines.

The incubation of 6-thiopurine metabolites in the presence of H_2O_2/HCO_3^- culminated in oxidizing these metabolites to different degrees. For instance, the incubation of 6-TX and 6-TUA in the presence of H_2O_2/HCO_3 resulted in a decrease of absorption for both metabolites with the appearance of a new peak centered at 375 nm (Fig. 4.2 A: C); however, there was a prominent decrease and shift in the absorbance of 6-TG ($\lambda = 340$ nm) without the appearance of a new peak, albeit isosbestic point is observed at 265 nm (Fig. 4.2 B).

The absorbance data plotted in Fig. 4.3A shows that there was no oxidation product in the reaction mixtures containing H_2O_2/HCO_3^- in the presence of AZA; however, 6-MP resulted in a prominent change in its absorbance, including gradually decrease in the peak at 319 nm with concomitant formation of a new peak at 272 nm (Fig. 4.3B). The kinetics of the oxidation of 6-MP was significantly affected by modulating the concentrations of both HCO_3^- (data not shown) and H_2O_2 and there was a degradation of the parent compound at 319 nm (Fig. 4.3C) that simultaneously was accompanied by forming a new product at 272 nm (Fig. 4.3D) with increasing the concentrations of H_2O_2. By taking together that unobservable change in the spectra of AZA; with the prominent change in the spectra of its metabolite 6-MP may suggest that sulfur atom in 6-MP play a pivotal role in its oxidation by H_2O_2/HCO_3^-.

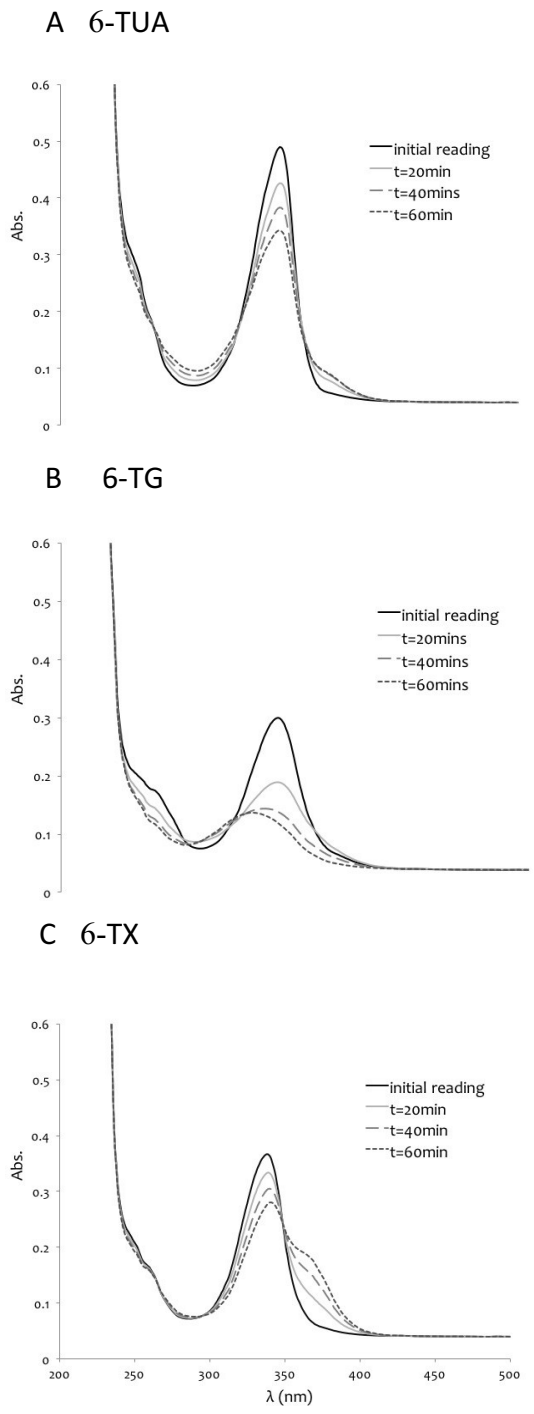


Figure 4.2 Spectrophotometric assay of H_2O_2/$NaHCO_3$-oxidized (HCO_4^-) 6-thiopurine metabolites. A spectrum was recorded from a reaction containing 1 mM H_2O_2, 25 mM $NaHCO_3$ and either 20 μM 6-TUA (A), or 20 μM 6-TG (B), or 20 μM 6-TX (C). The spectra were recorded immediately, at 0 and after 20 min. (solid

lines), and after 40, 60 min. (dashed lines). All reactions were carried out in 0.1M phosphate buffer containing 100 μM DTPA, pH 7.4, at room temperature. (n=3).

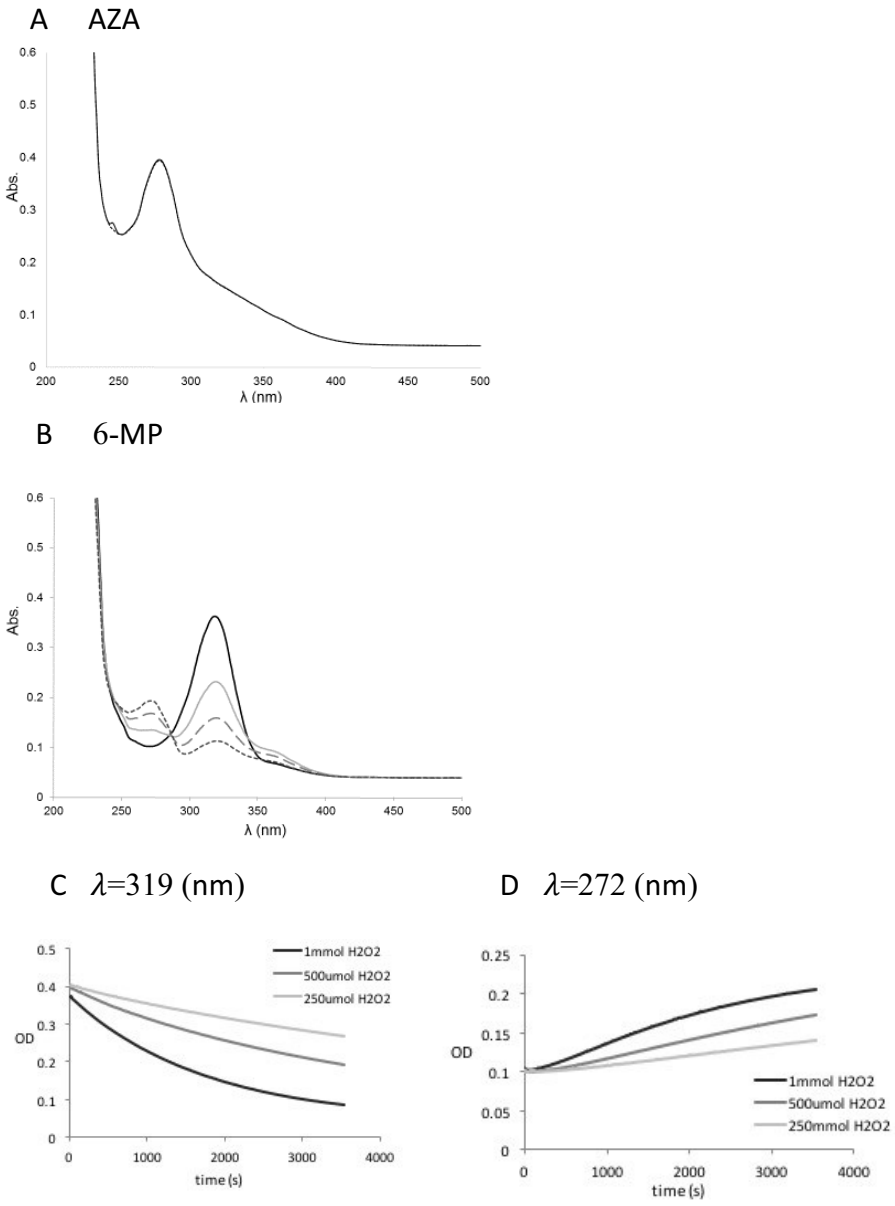


Figure 4.3 H_2O_2/$NaHCO_3$ catalyzes the oxidation of 6-MP oxidation, but not azathioprine. UV spectra of a reaction containing 1mM H_2O_2, 25 mM $NaHCO_3$ and either 20 µM AZA (A), or 20 µM 6-MP (B). These reactions were recorded immediately, after 20,40,60 min. Kinetic spectra were carried out for the reaction containing 20µM 6MP, 25mM NaHCO3 and different concentrations of H_2O_2 (250 µM, 500 µM, 1 mM) at 319 nm (C) and 272 nm (D). All reactions were carried out in 0.1M phosphate buffer containing 100 µM DTPA, pH 7.4, at room temperature. (n=3).

4.4.2 SOD peroxidase activity-catalyzed 6-MP oxidation.

The oxidation of 6-MP by SOD peroxidase activity resulted in a major product peak corresponding to an m/z of 182.9981 ($C_5H_4N_4O_2S$), which corresponds to 6-MP sulfoxide (Fig. 4.4D). The latter product was not detected by the omission of either SOD (Fig. 4.4B) or H_2O_2 (Fig. 4.4C) from the reaction, suggesting that the sulfur atom in 6-MP was oxidized by SOD peroxidase-generated $CO_3^{\cdot-}$ radicals, which are known to oxidize various compounds such as sulfur compounds [19].

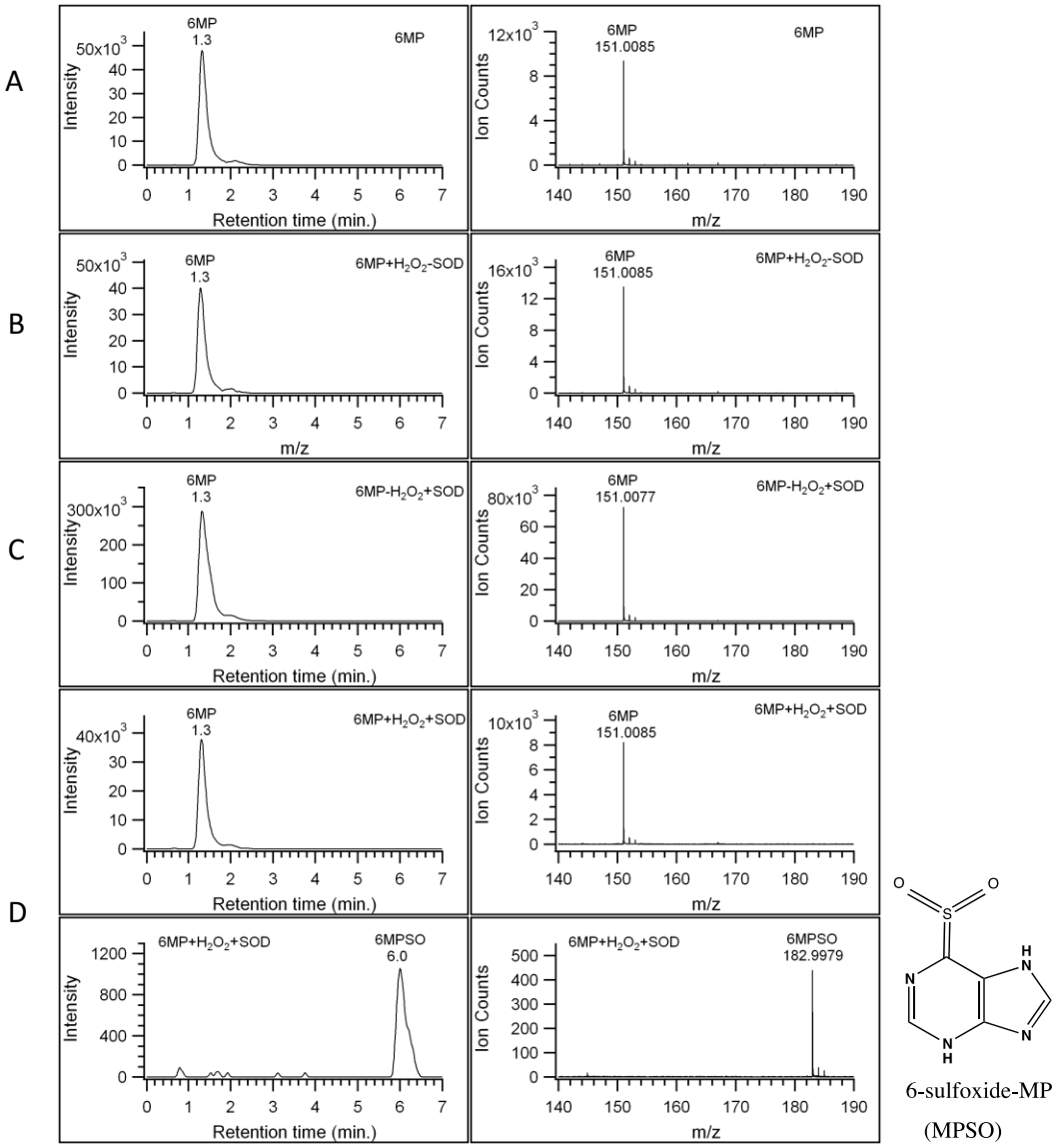


Figure 4.4 SOD peroxidase activity-catalyzed 6-MP oxidation. LC/MS of 6-MP metabolite produced from SOD peroxidase activity demonstrates the formation of 6-sulfoxide-MP. The reaction mixtures containing 33 mM HCO_3, 500 µM 6-MP, 1 mM H_2O_2, and 1 µM SOD (D). Either SOD-omitted (B) or H_2O_2-omitted (C) from reaction (D) resulted in abrogated 6-sulfoxide-MP formation. The chromatogram and mass spectrum of the corresponding peak demonstrated a major peak with only reaction (D): (D, rt = 6 min) 6-sulfoxide-MP (m/z 182.9979), and all the reactions (A, B, C, and D) demonstrated 6-MP peak (rt = 1.3 min, and m/z 151.0085). All reactions were carried out in 0.1M phosphate buffer containing 100 µM DTPA, pH 7.4, at room temperature. (n=2).

4.4.3 $CO_3^{\bullet-}$ produced from SOD peroxidase activity was scavenged by 6-MP but not AZA.

It is well documented that HCO_3^-/CO_2 (biological buffer system) enhances the SOD1 peroxidase activity through forming $CO_3^{\bullet-}$ radicals [20,21]. These radicals are strong oxidizing agents that are capable of oxidizing different compounds, including DMPO. It has been demonstrated that $CO_3^{\bullet-}$ radicals react with DMPO, at a rate constant of 2.5 x 10^6 M^{-1} S^{-1}, culminating in DMPO-OH which is due to the breakdown of the unstable DMPO-OCO_2 adduct, or possibly via DMPO oxidation to a radical cation ($DMPO^{\bullet+}$) that ultimately lead to DMPO-OH [25]. Interestingly, we found that the intensity of $CO_3^{\bullet-}$ radicals that resulted from reaction mixtures containing SOD, H_2O_2, and HCO_3, was significantly attenuated by increasing concentrations of 6-MP (Fig. 4.5 A (b-e)). The omission of SOD from the reaction in Fig. 4.5 A (b) resulted in a background signal (Fig. 4.5 A (e)). By comparing 6-MP to the prodrug, AZA, we found that the latter did not scavenge $CO_3^{\cdot-}$ radicals, albeit using high concentrations (Fig. 4.5 B (b)); however, 6-MP can react and scavenge $CO_3^{\cdot-}$ radicals, as judged by attenuating DMPO-OH adduct (Fig. 4.5 B (c)). This may indicate that $CO_3^{\cdot-}$ radicals could react with 6-MP at sulfur atom.

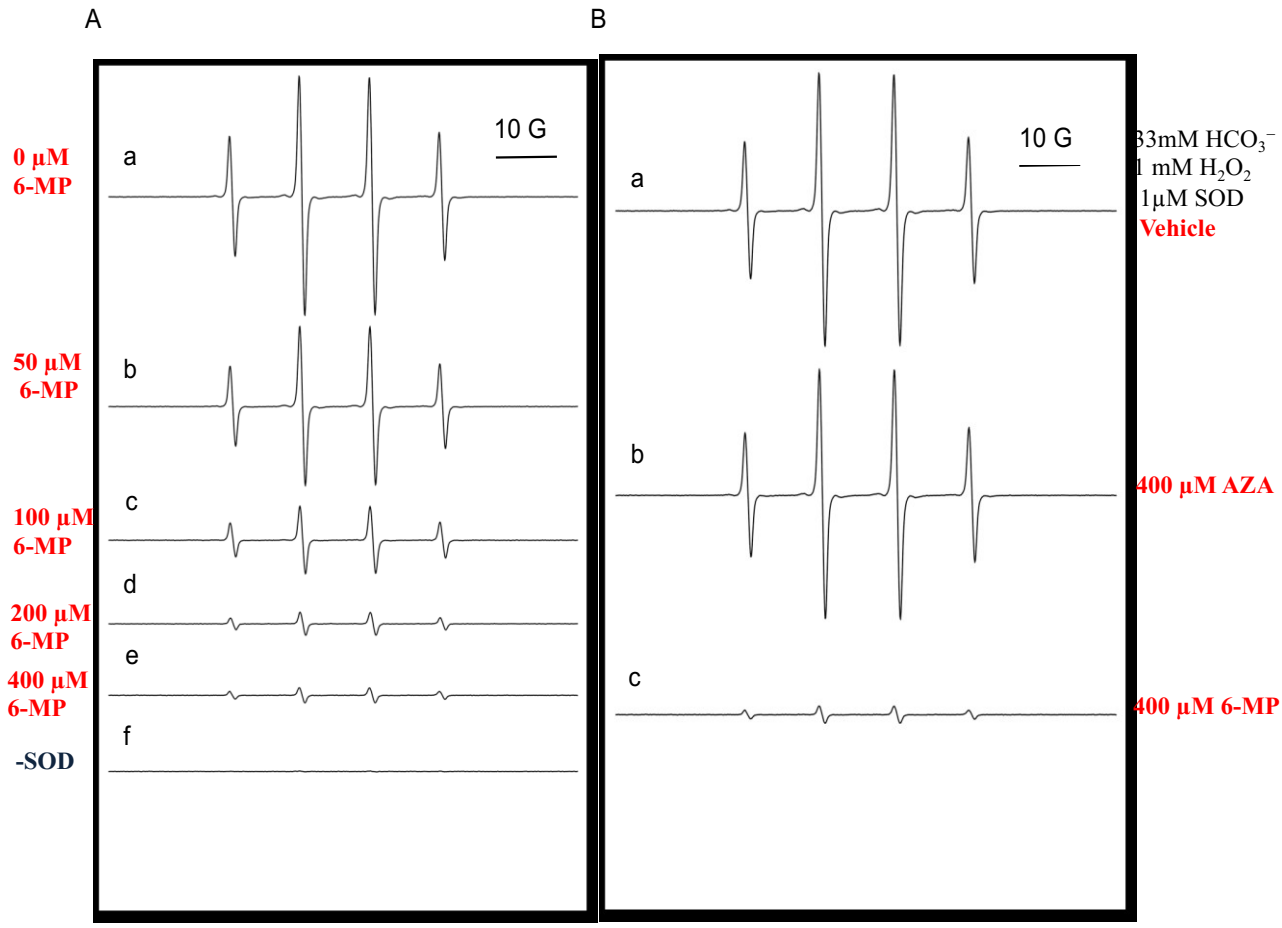


Figure 4.5 6-MP but not azathioprine-attenuated SOD derived $CO_3^{\bullet-}$radicals. EPR spectra demonstrating a concentration-dependent attenuation of the DMPO$^{\bullet}$/OH adduct with 6-MP **(A).** The spectra were generated by the reaction of 100 mM DMPO, 33 mM HCO_3^-, 1 mM H_2O_2 and 1 µM SOD with 0 µM 6-MP (a), 50 µM 6-MP (b), 100 µM 6-MP (c), 200 µM 6-MP (d) and 400 µM 6-MP (e). The omission of SOD (f) from reaction (b) showed no spectrum. The comparison effect of azathioprine and 6-MP on DMPO/$^{\bullet}$OH adduct **(B)**. A reaction containing 100 mM DMPO, 33 mM HCO_3^-, 1 mM H_2O_2 and 1 µM SOD with either vehicle (a) or 400µM azathioprine (b) or 400 µM 6-MP (c) are shown. All reactions were carried out using a 200 µL volume containing 0.1M phosphate buffer containing 100 µM DTPA, pH 7.4, at room temperature.

4.4.4 The incubation of 6-MP with SOD/H_2O_2/HCO_3 ameliorates SOD protein damage and inactivation.

It has been reported that tryptophan is a preferred target of $CO_3^{\bullet-}$ radicals, (rate constant 7 x 10^8 $M^{-1}S^{-1}$) [19]. The oxidation of hSOD1 by $CO_3^{\bullet-}$ could culminate in the formation of N-formyl-kynurenine and kynurenine, yielding covalent dimerization of hSOD1, which can be inhibited by tempol (a radical scavenger) [26]. In our study, we found that $CO_3^{\bullet-}$ radicals significantly inactivated SOD1; however, an increase in the concentrations of 6-MP resulted in ameliorated SOD1 activity (Fig. 4.6). Particularly, the concentrations of 6-MP (450, 600, and 750 μM) significantly inhibited the deleterious effect of $CO_3^{\bullet-}$ radicals on SOD1 activity (Fig. 4.6). Interestingly, 750 μM AZA did not ameliorate SOD1 activity as compared to 6-MP (Fig. 4.6), suggesting that sulfur atom in 6-MP play an essential role in scavenging $CO_3^{\bullet-}$ radicals.

To confirm the role of 6-MP in ameliorating SOD activity, we performed denaturing polyacrylamide gel electrophoresis as described in Materials and Methods to determine changes in the molecular mass distribution of bSOD 1 (Fig. 4.7). Our findings are comparable with other studies that showed an apparent molecular mass of bSOD 1 is 55-64 kDa [27,28]. Both AZA and 6-MP did not affect bSOD 1 in the absence of H_2O_2/HCO_3 (Lanes 1,2). The inclusion of H_2O_2/HCO_3^- with the absence of either 6-MP or AZA resulted in an attenuated protein band concomitant with the dissociation of the dimer of b SOD1 to monomer (Lanes 3,4). Interestingly, densitometric analysis of protein showed that an increase in the concentrations of 6-MP culminated in significantly reformed the protein band (Lanes 5-8), and the

prominent effect on bSOD 1 protein was observed with 6-MP as compared to AZA (Lanes 8,9). This may suggest that 6-MP has an ability to scavenge $CO_3^{\bullet-}$ radicals and subsequently to protect bSOD 1 protein.

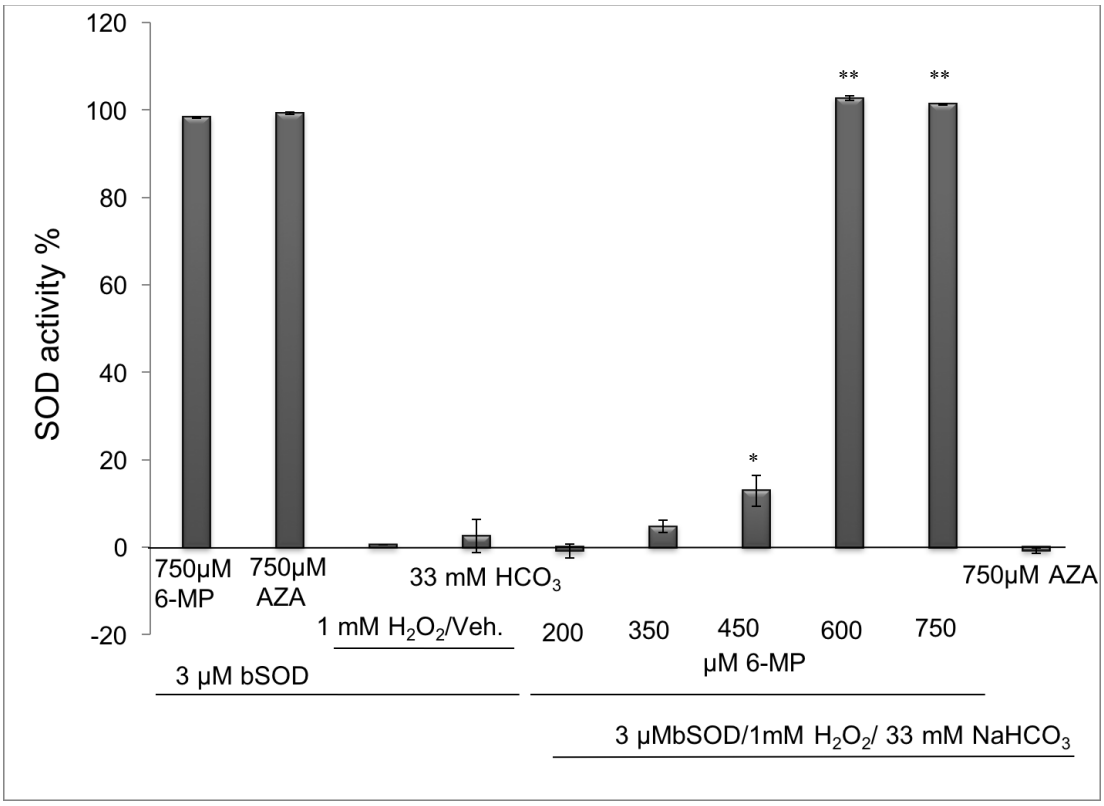
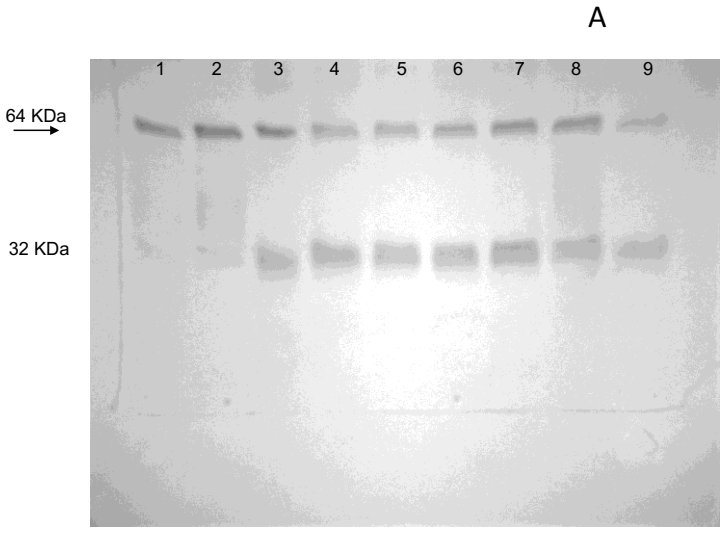


Figure 4.6 Effect of 6-MP and azathioprine on SOD activity. 3 µM SOD (2.7 mg/Ml) was incubated with a mixture containing 33 mM HCO_3, 1 mM H_2O_2, and either different concentrations of 6-MP (200, 350, 450, 600, and 750 µM) or 750 µM azathioprine for 6 h at 37 ^{O}C. After the reaction was stopped by catalase, bSOD1 dismutase activity was measured as described in the Materials and methods section. All reactions were carried out according to the manufacturer's instructions. Values are the means ± SD, ** $P<0.001$, * $P<0.05$. (n=3).



Lane	1	2	3	4	5	6	7	8	9
3μM SOD	+	+	+	+	+	+	+	+	+
6-MP (mM)	0.75	-	-	-	0.3	0.45	0.6	0.75	-
AZA (mM)	-	0.75	-	-	-	-	-	-	0.75
1 mM H_2O_2	-	-	+	+	+	+	+	+	+
33 mM HCO_3^-	-	-	-	+	+	+	+	+	+

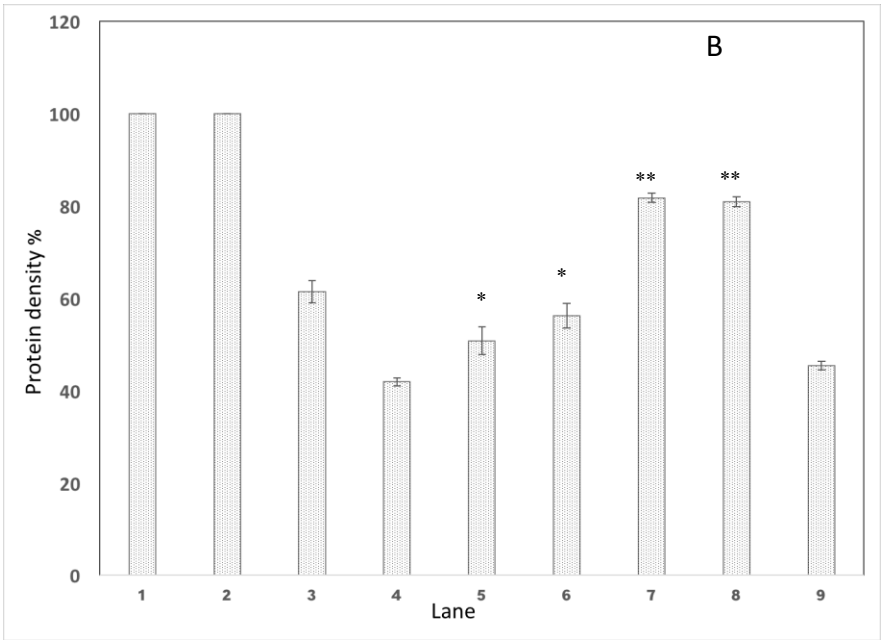


Figure 4.7 Effect of 6-MP and azathioprine on SOD protein. SDS-PAGE stained with Coomassie Blue (A). 3 μM SOD (2.7 mg/Ml) was incubated with a mixture containing 33 mM HCO_3, 1 mM H_2O_2, and either different concentrations of 6-MP

(200, 350, 450, 600, and 750 μM) or 750 μM azathioprine at 37 °C for 6 h. The arrow indicates bands that were quantified. The densitometric analysis of dimer of the b SOD1 band (at 64 KDa) (B). SOD1 separation was performed and all the reactions were carried out in 0.1M phosphate buffer containing 100 μM DTPA, pH 7.4. Values are the means ± SD, ** $P<0.001$, * $P<0.05$ as compared to Lane 4. (n=2).

4.4.5 The pre-incubation of 6-MP with H_2O_2/HCO_3-inactivated 6-MP cytotoxicity.

It has been reported that an increase in the concentrations of 6-thiopurines paradoxically resulted in decreased in their cytotoxicity [1-3]. In agreement with these studies, we found that high concentrations of 6-MP (0.5, 1, 2 mM) did not show a cytotoxic effect on HepG2 cells, and the cytotoxic effect of H_2O_2 (2 mM) on HepG2 was attenuated by 2 mM 6-MP (Fig. A1.7). In Fig. 4.8, preincubation of different concentrations of 6-MP with H_2O_2 is the essential step for inactivating the cytotoxic effect of 6-MP on both human hepatocellular carcinoma cells (HepG2) (A), and human embryonic kidney 293 cells (HEK293) (B). All the reactions were incubated under the same condition as described in Materials and methods. H_2O_2-omitted from reaction mixtures containing HCO_3^- and different concentrations of 6-MP culminated in significantly decreased in the metabolic activity of both cell lines (HepG2, A) and (HEK293, B); however, the preincubation of 6-MP with H_2O_2/HCO_3 resulted in a significantly attenuated the deleterious effect of 6-MP on the metabolic activity of both cells. This may suggest that bicarbonate activated system oxidized 6-MP, leading to inactivate 6-MP and subsequently decrease its cytotoxic effect.

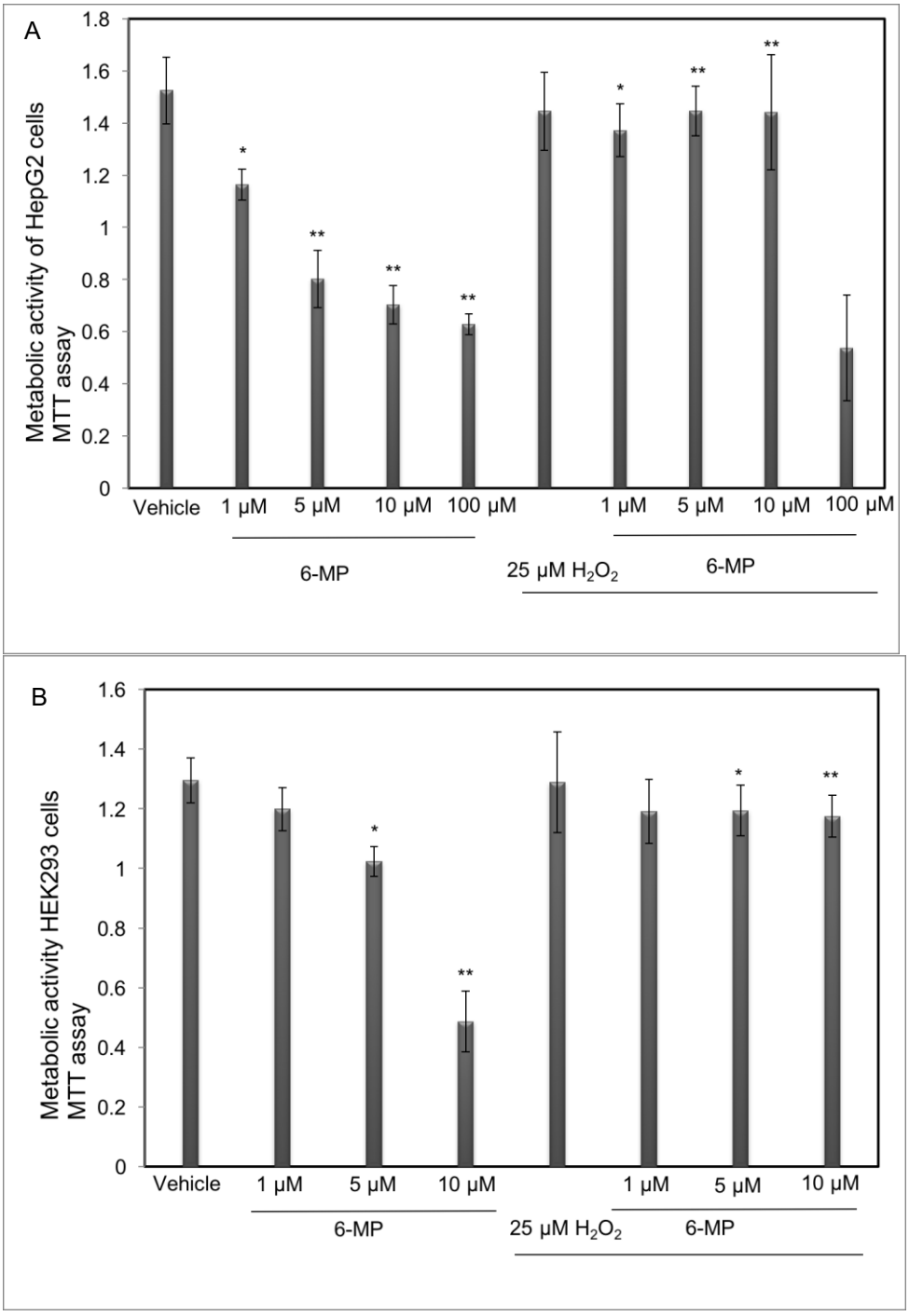


Figure 4.8 Effect of 6-MP, H_2O_2 and the combination of 6-MP and H_2O_2 on the metabolic activity of HepG2 (A) and HEK293 (B) cells. Cells were incubated in the presence of different concentrations of 6-MP (1, 5, 10, 100 μM) either with the presence or absence of 25 μM H_2O_2 that was preincubated with HCO_3^- and drug for 60 min. prior to adding it to cells as described in Experimental section. The

metabolic activity of HepG2 and HEK293 cells was determined using tetrazolium–based assay (MTT) at 72 h. Similar results were obtained in three different experiments. Values are the means ± SD (6-8 wells), ** $P<0.001$, * $P<0.05$ as compared to the preincubation of 6-MP with H_2O_2. (n=3).

4.5 Discussion

In this study, we investigated the involvement of H_2O_2/HCO_3^- (HCO_4^-) and SOD-peroxidase activity ($CO_3^{\bullet-}$) in oxidizing 6-MP, which is the first metabolite of azathioprine. The cytotoxicity of 6-thiopurine was paradoxically decreased by increased concentrations of these drugs that either repress the incorporation of 6-thiopurine into DNA through blocking the cell cycle or deplete intracellular ATP that is essential for the conversion of thioxanthosine monophosphate (TXMP) to thioguanosine monophosphate (TGMP) [1-3]. This may suggest that there may yet be other mechanisms by which 6-thiopurine cytotoxicity is decreased. Interestingly, our data showed that the oxidation of 6-MP by H_2O_2/HCO_3^- that catalyzed by SOD peroxidase activity could culminate in 6-MP sulfoxide formation, which inactivates the cytotoxic effect of 6-MP in both normal and cancer cell lines and consequently ameliorates both SOD protein and activity. The mechanistic pathway of oxidizing 6-MP (enzymatically or non-enzymatically) is depicted in Figure 4.9.

It has been proposed that guanine sulphinate (G^{SO2}), which is a major stable product of oxidizing free 6-TG base, and of 6-TG in DNA, could be also oxidized by either UVA exposure or treatment with mild oxidizing agent, monoperoxyphthalate (MMPP), leading to guanine sulphonate (G^{SO3}) formation, which is known to be refractory to further oxidation [4]. It is of interest to note that $CO_3^{\bullet-}$ radical is also mediated by the oxidation of guanine in DNA by a one-electron transfer process that results in the formation of stable guanine oxidation products [5]. It is well documented that $CO_3^{\cdot-}$ can be formed by different pathways, including SOD1 peroxidase activity [6-9].

Our measurements reported here indicate that there were notable changes in the spectra of 6-MP, 6-TX, 6-TUA and 6-TG after oxidation by H_2O_2/HCO_3^-, and the most prominent changes were observed with both 6-TG (Fig. 4.2 (b)) and 6-MP (Fig. 4.3 (b)). It is pertinent to point out that HCO_3^- is proposed as the effective activator for H_2O_2 to oxidize sulfides in mixed *tert*-butyl alcohol/water solvent [10], which is in agreement with our findings that 1 mM of H_2O_2 and 25 mM HCO_3 showed the maximum effect on oxidizing 6-MP in a short period of time (Figure 4.3-C and D). Richardson et al. reported that the BAP (bicarbonate-activated peroxide) oxidation system can oxidize sulfides, and can also catalyze the formation of methionine sulfoxide [11,12]; however, the concentrations of both H_2O_2 (0.2-4 M) and HCO_3^- (0.1 M) are very high as compared to those in our study. Our results suggest that H_2O_2/HCO_3 oxidized the sulfur atom in 6-thiopurines as evidenced by the unchanged spectra of AZA. It has been reported that a high concentration of H_2O_2 in the presence of acetic acid, which results in peroxyacid formation [11], catalyzed the oxidation of 6-TG, yielding S-oxide-thioguanine [13], further implicating the sulfur atom as the main site for oxidizing 6-thiopurines.

The spectra of 6-TG were dramatically declined by H_2O_2/HCO_3^- without a concomitant increase in forming new products as compared to the oxidation of 6-MP. These findings are comparable with another study which showed that oxidizing 6-TG (thio group) by electrochemical oxidation led to form 6-TG thiyl radicals, culminating in dimeric product [14]. Regino et al. showed that the oxidation of disulfide by H_2O_2 was catalyzed by HCO_3^-, leading to both thiosulfonate and sulfonate formation [15]. Also, it has been reported that HCO_4^- could oxidize tertiary and secondary amines that resulted in amine oxides and nitrones, respectively [16].

This may suggest that H_2O_2/HCO_3^- could oxidize 6-TG at a sulfur atom, yielding to potentially 6-TG sulfoxide as evidenced by the slightly change in forming new peak compared to 6-MP (λ 275 nm); however, the exocyclic amino group in 6-TG could potentially enhance 6-TG disulfide dimer formation.

It is well known that the degradation or inactivation of 6-MP by xanthine oxidase results in forming 6-TX and subsequently 6-TUA [17]. The latter product can be oxidized to 6-TUA diimine, yielding 6-TUA diol [18]. Our findings suggest that the presence of hydroxyl group in 6-TX and 6-TUA could potentially modulate the oxidizing effect of H_2O_2/HCO_3^- on sulfur atom as compared to 6-MP. Further studies are required to investigate the role of inductive effect in oxidizing sulfur atom by H_2O_2/HCO_3^-.

We used LC/MS analysis to detect the product of oxidizing 6-MP by both H_2O_2/HCO_3^- and SOD-generated $CO_3^{\bullet-}$ system. Interestingly, we found that SOD peroxidase activity catalyzed the oxidation of 6-MP at the short period of time (30 min.) compared to H_2O_2/HCO_3^- (24 h); both reaction, however, culminated in a sulfoxide product ($C_5H_4N_4O_2S$ 182.9981 m/z) (Fig. 4.4). Our results are comparable with another study that showed the oxidation of 6-thioxanthine eventually results in forming a sulfinic acid (RSO_2^-) after more than 14 h [18], suggesting that H_2O_2/HCO_3^- could oxidize 6-MP at 24 h; however, to eradicate any artifact, we used SOD-peroxidase generated $CO_3^{\bullet-}$ radical. The latter radical is known to oxidize various compounds, including phenols, anilines, and sulfur compounds as well as amino acids such as histidine and tryptophan at different rate constants; $CO_3^{\bullet-}$ reacts with its target by either hydrogen abstraction or oxygen atom /oxide transfer [19].

To confirm the role of SOD peroxidase activity in oxidizing 6-MP, we utilized EPR. The EPR is a sensitive and definitive technique and mainly used to detect and measure short-lived free radicals that need to be stabilized via spin traps. In this study, we utilized DMPO as a spin trap to stabilize the $CO_3^{\bullet-}$ radical. Our finding is in an agreement with other studies that showed the incubation DMPO with SOD peroxidase-generated $CO_3^{\bullet-}$ radicals could result in forming DMPO/$^{\bullet}$OH adducts [20,21]. It is of interest to note that the rate constant of the reaction of $CO_3^{\bullet-}$ radical with itself is relatively high ($k=3x10^7$ $M^{-1}.S^{-1}$) [19], and thus we used 100 mM DMPO to trap and stabilize this radical. Interestingly, we found that there was a decrease in the intensity of $CO_3^{\bullet-}$ (DMPO/$^{\bullet}$OH) in the presence of 6-MP but not AZA (Figure 4.5), suggesting that the sulfur atom in 6-MP could be oxidized by $CO_3^{\bullet-}$ through either electron transfer or hydrogen abstraction, resulting in potentially thiyl radicals that rapidly react with O_2 to form peroxyl radicals ($RSOO^{\bullet}$) [19,22]. However, in our findings, we cannot detect thiyl radicals as the concentrations of 6-MP are very low (50-400 μM) compared to the previous study that showed millimoles concentrations of 6-MP [23]. Thiyl radicals exist in an equilibrium with $RSOO^{\bullet}$, which may explain why we did not detect these radicals. Moore et al. showed that the irradiation of 6-MP resulted in forming thiyl radical that does not reduce DMPO, but it can reduce both 2-methyl-2-nitrosopropane (MNP) and nitromethane (NM), yielding MNP/$^{\bullet}$H and $CH_3NO_2^{\bullet-}$, respectively [23]. By taking together that the formation of 6-MP sulfoxide and the attenuation of $CO_3^{\bullet-}$ intensity by 6-MP as evidenced by LC/MS and EPR measurements, respectively, may suggest

that SOD peroxidase-generated $CO_3^{\bullet-}$ oxidized 6-MP at the sulfur atom and subsequently led to form 6-MP sulfoxide.

We carried out further investigation to determine the effect of oxidizing 6-MP on both SOD protein and activity. Interestingly, 6-MP concentration-dependently ameliorated SOD protein and activity from the damaging effect of $CO_3^{\bullet-}$ radicals; however, azathioprine did not protect both SOD protein and activity (Fig. 4.6 and 4.7). This may suggest that the formation of 6-sufoxide-MP could potentially ameliorate SOD protein damage and inactivation as it has been reported that dimethyl sulfoxide has an ability to ameliorate SOD activity in the mouse liver after exposed to the irradiation [24]. Our findings are comparable with the study that showed both H_2O_2 and HCO_3^- decreased human SOD1 [26].

Also, we found that H_2O_2 could cause a dissociation of the dimer of bSOD1to monomer that is in agreement with the study that showed bSOD1 concentration, temperature, and urea could be contributed to the dissociation of bSOD1 [40]. Furthermore, our findings are also in agreement with a study that showed the exposure of SOD to HOCl resulted in dissociation of protein band into units of 16 kDa [28]. Queiroz et al. showed that tempol could protect SOD from the oxidation by $CO_3^{\bullet-}$ [26]; this is in agreement with our study that showed the 6-MP protected SOD from the oxidation by $CO_3^{\bullet-}$ in a concentration-dependent manner (Fig. 4.7). That may suggest that 6-MP can be oxidized by $CO_3^{\bullet-}$, leading to 6-sulfoxide-MP that ameliorated both SOD protein damage and inactivation.

We also demonstrated the effect of preincubation of H_2O_2 and HCO_3^- (bicarbonate activated peroxide system) with 6-MP on its cytotoxicity by using both HepG2 and

HEK293 cells (Fig. 4.8). The HepG2 cells were selected as these cells characterized by secretion of SOD1; and low levels of catalase, which potentially results in accumulation of H_2O_2 [29,30], suggesting that SOD1 peroxidase-generated oxidative stress can be initiated. The HEK293 cells were selected as a positive control since 6-MP cytotoxic activity in these cells has been established (personal communication, Dr. Elaine Leslie). Our findings indicate that the bicarbonate-activated peroxide system appears to have a pivotal role in oxidizing 6-MP at the sulfur atom and consequently leads to inactivate the cytotoxic effect of 6-MP on both human cell lines.

In conclusion, our results show that both bicarbonate-activated peroxide system (HCO_4^- from H_2O_2/HCO_3^-), and SOD peroxidase-generated $CO_3^{\bullet-}$ culminate in oxidizing 6-MP that subsequently lead to form 6-sulfoxide-MP, which inactivates 6-MP's cytotoxic effect on both normal and cancer cell lines, and ameliorates SOD protein and activity by using a biochemical system. Clearly, the free sulfur atom plays a pivotal role in oxidizing 6-MP. Further studies are required to identify the effect of oxidizing 6-MP on the cellular antioxidant enzymes, and to reveal the molecular pathways involved in modulating these enzymes and causing a decrease in the cytotoxic effect of 6-MP on both HepG2 and HEK293 cells.

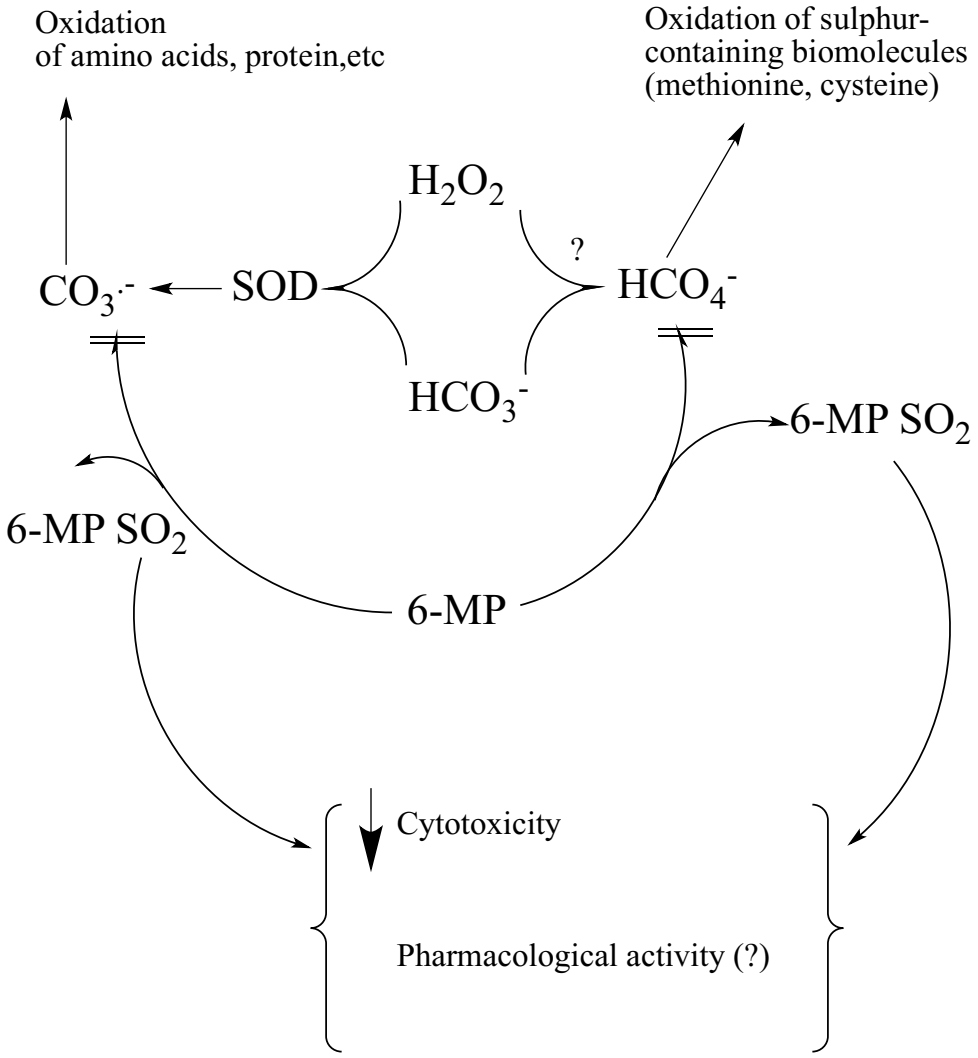


Figure 4.9 Proposed mechanistic pathway of oxidizing 6-MP by SOD peroxidase activity and HCO_4^-. 6-MP can be oxidized by carbonate radical ($CO_3^{\cdot -}$), a product of SOD peroxidase activity, and bicarbonate-activated peroxide system (potential HCO_4^-) culminating in 6-sulfoxide-MP (6-MP SO_2). The latter product may inactive 6-MP's cytotoxicity and ameliorate b SOD1 protein damage and inactivation.

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Chapter 5

General conclusion and future directions

5.1 General conclusions

This is the first study that has shown the oxidation of drugs by SOD-peroxidase-generated oxidative stress could potentially culminate in the modulation of their cellular cytotoxic effects. Particularly, we presented here the ability of SOD peroxidase-generated $CO_3^{\bullet-}$ to oxidize both PBZ and 6-MP, leading to form 4-OH-PBZ and 6-sulfoxide-MP, respectively.

Our findings emphasize the importance of two fundamental chemical classes of drugs in these reactions. The diketone moiety of PBZ has a pivotal role in scavenging $CO_3^{\bullet-}$ as evidenced by forming PBZ carbon-centered radicals, which in turn, react with oxygen molecule and GSH to form both 4-hydroperoxy-PBZ/4-hydroxy-PBZ and thiyl radicals, respectively. The latter potentially enhanced PBZ's cytotoxicity. In addition, it is widely recognized that ROS could contribute to oxidize thiol-containing compounds. Interestingly, these results emphasize the key role of the bicarbonate-activated peroxide system (H_2O_2/HCO_3^-) in oxidizing 6-thiopurines, particularly 6-MP that can be catalyzed by the presence of SOD-peroxidase generated $CO_3^{\bullet-}$, culminating in 6-sulfoxide-MP that inactivates 6-MP's cytotoxicity in both cancer and normal cell lines and consequently ameliorates SOD activity.

Despite the numerous studies that have shown the role of cytochrome P450 in metabolizing drugs or new chemical entities [1], there is still a scarcity in studying the effect of peroxidase enzymes on drugs metabolism and their cytotoxicity. Thus, this thesis attends to propose and justify the role of SOD peroxidase-derived $CO_3^{\bullet-}$ in oxidizing drugs (PBZ, 6-MP) that are used in chronic inflammation as well as to reveal the effect of oxidizing these drugs on their toxicity.

Superoxide dismutases (SODs) are essential enzymes in cells that are present in three major isoforms, including SOD1 (CuZn-SOD), which is located in both the cytosol and in the intermembrane space of mitochondria, SOD2 (MnSOD), which is present in the matrix of mitochondria, and SOD3 (CuZn-SOD) that is located in the extracellular space of cells [2,3]. SODs act catalytically to convert superoxide anion radical ($O_2^{\bullet-}$) to hydrogen peroxide (H_2O_2) and O_2 [4]. It has been demonstrated that H_2O_2 has an ability to inactivate SOD1, culminating in a localized hydroxyl radical (SOD-OH); this process is known as a peroxidase activity of SOD1. For instance, Cu (II) in the active site of SOD1 would be reduced by H_2O_2 to form Cu (I) that in turn will react with H_2O_2 to generate Cu^{+2}- bound OH. The SOD-OH in the presence of a substrate such as HCO_3^-/CO_2 (a biological buffer) will generate oxidative stress, including $CO_3^{\bullet-}$ [5-7].

There is a strong link between cancer and oxidative stress. At the site of inflammation, cells such as mast cells and leukocytes are recruited to the site of damage, culminating in stable elevation of reactive oxygen species (ROS) and consequently oxidative stress [8,9]. The imbalance between the production of free radicals (ROS, reactive nitrogen species (RNS)) and their clearance by antioxidant systems results in oxidative stress formation. For instance, the reduction of antioxidant defenses like antioxidant enzymes (such as superoxide dismutases, catalyses, glutathione peroxidases) and elevation of O_2 in the presence of radical-forming xenobiotics could result in both oxidative and nitrosative stress formation. That may suggest that altering cellular homeostasis could foster genomic instability and consequently raise the risk of cancer development [10,11].

It is pertinent to point out that the $CO_3^{\bullet-}$ can also be generated from various sources. Remarkably, $O_2^{\bullet-}$ is highly reactive towards SOD ($k = 1.6 \times 10^9\ M^{-1}.\ S^{-1}$) and nitric oxide ($NO^{\bullet}$) ($k = 5 \times 10^9\ M^{-1}.\ S^{-1}$) to generate both oxidative stress (H_2O_2) and nitrosative stress, such as peroxynitrite ($ONOO^-$) [10,12]. The H_2O_2 can indirectly lead to the formation of highly-damaging free radicals, $HO^{\bullet}$, through the well-known Harber-Weiss as well as the iron-catalyzed Fenton reactions that consequently in the presence of bicarbonate could culminate in $CO_3^{\bullet-}$ formation [12]. Also, the homolytic dissociation of peroxymonocarbonate, which is the product of reaction CO_2 with $ONOO^-$, could lead to generating $CO_3^{\bullet-}$. It has been shown that xanthine oxidase with the presence of acetaldehyde and bicarbonate could result in $CO_3^{\bullet-}$ formation using spectrometry studies [12,13].

Our findings in this study show that SOD peroxide-derived $CO_3^{\bullet-}$ catalyzed the oxidation of both PBZ and 6-MP. PBZ was prescribed to treat osteoarthritis, however, this drug is no longer available because of its cytotoxic effects, including hepatotoxicity, renal failure and agranulocytosis [14-16]. The exact mechanism by which PBZ induced these deleterious side effects is still elusive. From the chemical point of view, PBZ has an ability to scavenge ROS because it possesses a diketone moiety in its structure and it has been reported that PBZ radicals can be generated in the presence of horseradish peroxidase (HRP, a plant peroxidase) [17]. Thus, PBZ was selected in this work as a candidate for pyrazolidine diketone drugs in order to investigate the role of SOD peroxidase-derived oxidative stress in oxidizing PBZ and subsequently the association of SOD peroxidase activity in its toxicity. On the other hand, 6-MP is a prodrug that requires bioactivation to exert its therapeutic effect

against diseases including leukemia and IBD (Crohn's and ulcerative colitis) [18]. It is believed that the inflammation sites, as well as tumor tissues, are characterized by the generation of ROS that are apparently associated with oxidizing thiol-containing compounds. For instance, it has been shown that cells that are involved in the host-defense response, such as polymorphonuclear leukocytes that contribute to ROS production, promote endothelial dysfunction by oxidation of thiol group in crucial signaling proteins such as tyrosine phosphatases [8,9]. Nonetheless, 6-MP was selected in this study as a candidate of thiopurine derivatives to investigate the role of SOD via generation $CO_3^{\bullet-}$ in oxidizing this drug and to determine the effect of bicarbonate-activated peroxide (BAP) that is well-known as SOD peroxidase initiation on 6-MP's cytotoxicity. Furthermore, human hepatocellular carcinoma (HepG2) cells were selected in this study to reveal the effect of BAP as well as SOD peroxidase activity on the toxicity of both PBZ and 6-MP. The HepG2 cells show altered in antioxidant enzyme levels that are involved in the control of the redox state, namely (i) diminished catalase activity; (ii) increased secretion of SOD1 [19,20]. This may suggest that there is a chance for accumulation of H_2O_2, which apparently initiates both BAP system and SOD peroxidase activity.

The results in this thesis indicate that SOD peroxidase-derived $CO_3^{\bullet-}$ is capable of oxidizing both PBZ and 6-MP, culminating in 4-hydroxy-PBZ and 6-sulfoxide-MP, respectively. First of all, our findings confirm that SOD1 peroxidase activity is comparable with other studies that showed peroxidases, including PGHS hydroperoxidase and HRP, although different spin traps were used in those studies [21-24]. Although PBZ showed a concentration-dependent attenuation of $CO_3^{\bullet-}$ with

an increase in the PBZ carbon-centered radicals, there was a decrease in the intensity of $CO_3^{\bullet-}$ without detecting thiyl radicals. Also, the intensity of $CO_3^{\bullet-}$ was not affected by both 4-OH PBZ or azathioprine (AZA) that are used to confirm the specific sites for these reactions. This suggests that both PBZ (C_4 position) and 6-MP (sulfur atom) could be oxidized by $CO_3^{\bullet-}$ either through electron transfer or hydrogen abstraction [12], resulting in PBZ carbon-centered radicals, as evidenced by increasing oxygen uptake and detecting 4-hydroperoxy PBZ, and potentially 6-MP thiyl radicals albeit we cannot detect these radicals as the concentrations of 6-MP are very low (50-400 μM) compared to the previous study that showed photooxidation of millimolar concentrations of 6-MP [25]. It has been reported that thiyl radicals rapidly react with O_2 to form peroxyl radicals ($RSOO^{\bullet}$) [12,26]. Thiyl radicals exist in an equilibrium with $RSOO^{\cdot}$, which may explain why we did not detect these radicals. By taking together that the formation of 4-hydroxy PBZ as well as 6-MP sulfoxide and the attenuation of $CO_3^{\bullet-}$ intensity as judged by LC/MS and EPR measurements, may suggest that SOD peroxidase-derived $CO_3^{\bullet-}$ could oxidize medial carbon atom of the 1,3 diketone moiety in PBZ and sulfur atom in 6-MP.

It is pertinent to point out that $CO_3^{\bullet-}$ has an ability to oxidize various biotarget molecules, including SOD. It has been reported that $CO_3^{\bullet-}$ can oxidize tryptophan residue in human SOD (h SOD), leading to covalent dimerization of h SOD via the formation of N-formyl-kynurenine and kynurenine [27]. Thus, we investigated the effect of both PBZ and 6-MP on ameliorating SOD protein damage and inactivation as both drugs showed a concentration-dependent attenuation of the $CO_3^{\bullet-}$ intensity. Our results showed that there is a similarity regarding the effect of H_2O_2 on the

dissociation of bovine SOD1 (b SOD1), which is in an agreement with other studies that showed b SOD concentrations and temperature could affect the dissociation of a dimer of b SOD1 [28]. Our results are comparable with the study that showed both H_2O_2 and HCO_3^- decreased human SOD1 (h SOD1) [27]. However, there is a difference between the effect of PBZ on ameliorating SOD protein and its activity compared to 6-MP. We found that PBZ did not protect b SOD1 from the deleterious effects of $CO_3^{\bullet-}$, while 6-MP showed a concentration-dependent protection of b SOD1 against $CO_3^{\cdot-}$. As PBZ scavenging of $CO_3^{\bullet-}$ resulted in a carbon-centered radical on PBZ, it is possible that the latter could also oxidize critical amino acids in SOD1, which may explain why PBZ did not ameliorate b SOD activity against $CO_3^{\bullet-}$ [29,30]. On the other hand, 6-MP showed a protective effect on b SOD1; this is in agreement with another study that showed tempol has the capability to protect h SOD1 from the oxidation of $CO_3^{\bullet-}$, suggesting that 6-MP can be oxidized by $CO_3^{\bullet-}$, leading to 6-sulfoxide-MP that ameliorated b SOD1 activity. It is pertinent to point out that dimethyl sulfoxide has an ability to ameliorate SOD activity in the mouse liver after exposed to the irradiations [31]. Nevertheless, forming PBZ carbon-centered radicals, which are relatively unstable and highly reactive towards proteins, and 6-sulfoxide-MP via oxidizing medial carbon atom of the 1,3 diketone moiety (C_4) in PBZ and sulfur atom in 6-MP by $CO_3^{\bullet-}$ may explain the contrast of the effect of PBZ and 6-MP on ameliorating b SOD1 protein damage and inactivation.

In order to investigate the biological relevance of the effect of BAP on the cytotoxicity of PBZ and 6-MP as well as the contribution of extracellular SOD to BAP's effect, we utilized HepG2 cells as a model in this study. We found that the

BAP system can affect the cytotoxicity of both PBZ and 6-MP differently. The PBZ cytotoxicity was enhanced by BAP system while the pre-incubation of 6-MP with BAP led to inactivate 6-MP cytotoxicity as judged by decreased metabolic activity of HepG2 cells. It is noteworthy that BAP in the presence of SOD could initiate SOD1 peroxidase activity and subsequently results in oxidative stress formation including $CO_3^{\bullet-}$ [5]. Although our findings showed PBZ carbon-centered radicals were formed upon addition PBZ and BAP via utilizing intact HepG2 cell and these radicals were attenuated by increasing the concentrations of DDC (a strong copper chelator and SOD1 inhibitor) as evidenced by EPR analysis, the main source of causing these radicals is still elusive as HepG2 cells contain different metalloenzymes and peroxidase enzymes. This may suggest that BAP with the presence of peroxidase enzymes including SOD1 could contribute to PBZ toxicity through increased PBZ carbon-centered radicals that are relatively unstable and highly reactive towards proteins. On the other hand, the cytotoxic effect of 6-MP on HepG2 cells (as these cells are characterized by secretion of SOD1; and low levels of catalase) and HEK293 cells (as a positive control) was significantly attenuated by the preincubation of 6-MP with BAP system, suggesting that oxidizing 6-MP at sulfur atom by both BAP and potentially SOD peroxidase activity could lead to inactivate the cytotoxic effect of 6-MP on both cell lines. Our findings are in agreement with other studies that showed the cytotoxicity of 6-thiopurines was decreased by increased concentrations of these drugs [32-34].

The results of this study indicate that SOD peroxidase-derived $CO_3^{\bullet-}$ could possibly oxidize both PBZ and 6-MP and consequently modulate their cytotoxicity.

Based on our findings, SOD with a combination of other peroxidase enzymes and in the presence of an accumulation of H_2O_2, which apparently occurs in inflammatory tissues and cancers, could be potentially considered as a significant factor for modulating drugs toxicity, namely PBZ and 6-MP.

5.2 Future directions

In this work, we demonstrated that bovine SOD1 (b SOD1) peroxidase-generated $CO_3^{\bullet-}$ oxidized both PBZ [30] and 6-MP (in processing for publication), thus it is worthwhile to investigate the effect of human SOD1 (h SOD1) on oxidizing these drugs. Our preliminary findings showed that 1 μM h SOD1 could oxidize PBZ but not 4-OH-PBZ, culminating in PBZ carbon-centered radical formation (Fig. A1.1). We also found that the intensity of $CO_3^{\bullet-}$radical was higher in the presence of 1 μM h SOD1, as compared to 1 μM b SOD1. Furthermore, extracellular h SOD1 (0.15 μM) enhanced the cytotoxic effect of (PBZ/H_2O_2) as evidenced by decreased the metabolic activity of HepG2 cells (Chapter 3 (Fig. 3.11)) compared to b SOD1 (Fig. A1.2). These preliminary results may suggest that there is a difference between h SOD1 and b SOD1 regarding their peroxidase activity even though it has been shown that both bovine-SOD and human-SOD dismutase activity have a similar efficacy using an experimental rat model [35]. Thus, future studies should be performed to distinguish the catalytic potency of b SOD1 vs h SOD1 peroxidase systems as well as the contribution of these peroxidases in the molecular mechanism of PBZ induced-toxicity through utilizing cancer cells.

Isoniazid (INH) is a key drug against tuberculosis. In addition to its mechanism of action, INH can also modulate the microenvironment of SOD1without affecting its dismutase activity [36,37]. Our preliminary results showed that 4 mM INH was capable of decreasing the formation of both DMPO/$^{\bullet}$OH and DMPO/$^{\bullet}$PBZ adducts (Fig. A1.3). Although low concentrations of INH (0.1, 0.2, 0.5, 1, and 2 mM) did not attenuate DMPO/$^{\bullet}$OH adduct (data not shown), these results may suggest that high concentrations of INH are required to make a complex with the metals (Cu and Zn) in the active site of SOD1, that resulted in decreased SOD-OH formation and consequently repressed $CO_3^{\bullet-}$ generation. It is worthwhile to investigate the effect of INH on PBZ cytotoxicity through repressing SOD1 peroxidase-generated oxidative stress.

Additional preliminary results showed that SOD1 peroxidase-derived $CO_3^{\bullet-}$could catalyze the oxidation of both sulfinpyrazone (a sulfoxide-containing uricosuric drug) and aminoglutethimide (anti-steroid drug) to form carbon-centered radical (Fig. A1.4) and nitrogen-centered radical (Fig. A1.5), respectively. It is intriguing to do further investigations to screen other drugs that can be oxidized by SOD1 peroxidase activity such as etoposide (anti-cancer drug) and captopril (anti-hypertensive drug) to generate various radicals, including phenoxyl and thiyl radicals, respectively [38,39. In vitro studies, it is valuable to monitor the synergistic cytotoxic effect of combined extracellular SOD with drugs (sulfinpyrazone, aminoglutethimide, etoposide and captopril) on cancer cells (HepG2). Consequently, by using an in vivo study, the most cytotoxic combined drug (SOD/drug) can be used to investigate as a therapeutic

tool to target tumor cells i.e. beneficial side effect (by utilizing drugs' side effect in treating cancer diseases).

It is worthwhile to investigate the effect of both BAP system and SOD peroxidase activity on 6-thiopurines including 6-thioguanine (6-TG). Our preliminary findings showed that the preincubation of H_2O_2 and HCO_3^- with 6-TG significantly inactivated its cytotoxic effect as evidenced by decreased the metabolic activity of HEK293 cells (Fig. A1.6). Future studies are required to identify the effect of oxidizing both 6-MP and 6-TG on the cellular antioxidant enzymes, and to reveal the molecular pathways involved in modulating these enzymes and causing cells proliferation.

It is well known that doxorubicin (anti-cancer drug) exerts its effect in part via production of ROS; however, cardiotoxicity is the main side effect from utilizing this drug. In Fig. A1.7, we found that 6-MP could attenuate the cytotoxic effect of H_2O_2. From the reactivity point of view, we can envision that combined (DOX/6-MP) could potentially ameliorate the deleterious effect of DOX on cardiomyocytes.

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Appendix 1

Supplementary Experimental Data

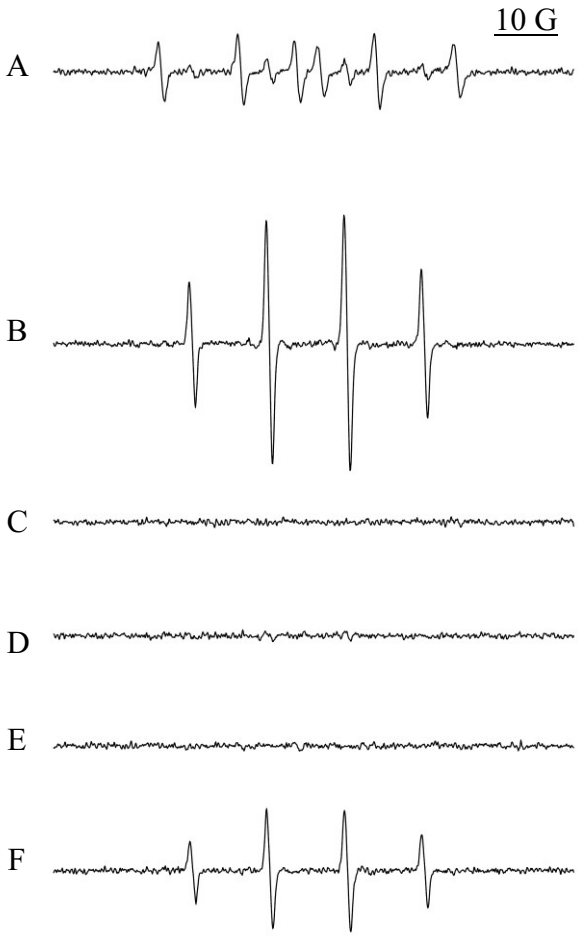

Figure A1.1 Effect of human SOD peroxidase activity on PBZ and 4-OH PBZ. All reactions were carried out using a 200 µL volume containing 0.1M phosphate buffer containing 100 µM DTPA, pH 7.4, at room temperature. The spectra were generated by the reaction of 100 mM DMPO, 33 mM HCO_3^-, 25 mM glucose, 7.5 U glucose oxidase and 1 µM h SOD with 1 mM PBZ (A), 0 µM PBZ (B), 1 mM 4-OH-PBZ (F). The omission of either SOD (C) or glucose/glucose oxidase (D) or HCO_3^- (E) from reaction (A) showed no free radical detection in the spectrum.

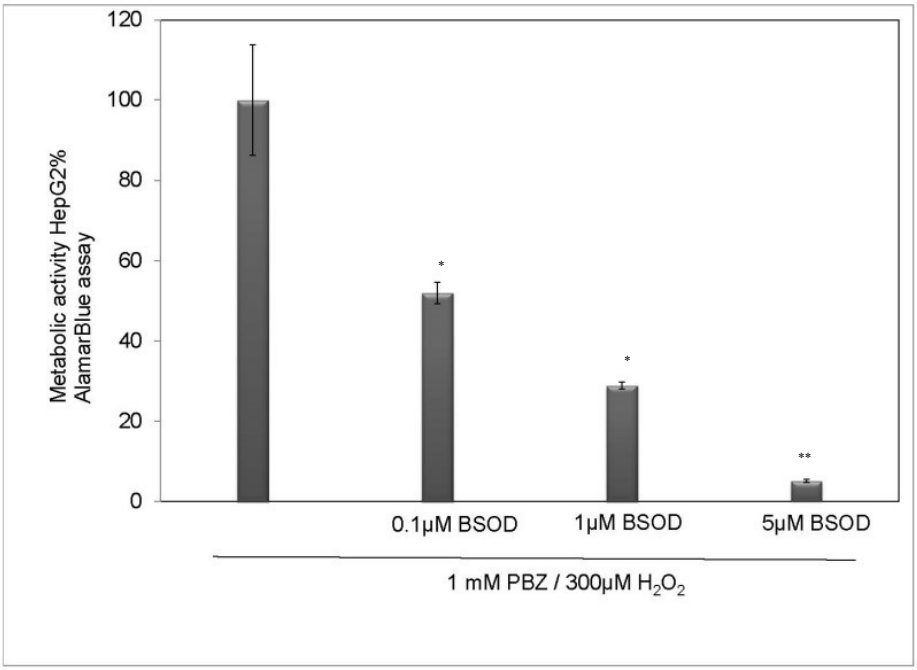

Figure A1.2 Effect of extracellular bSOD1 on the cytotoxic effect of the combined treatment PBZ/H_2O_2. HepG2 cells were treated with combined 1mMPBZ/300 µM H_2O_2 in the absence or presence different concentrations of bSOD1 and incubated for 24 h. The metabolic activity of HepG2 was determined by using alamarBlue assay.

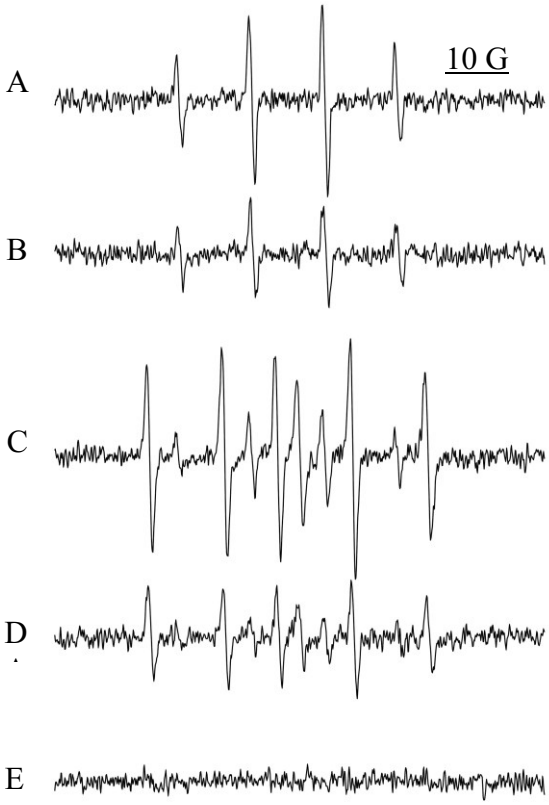

Figure A1.3 Effect of INH on DMPO/$^{\bullet}$OH and DMPO/$^{\bullet}$PBZ adducts. All reactions were carried out using a 200 μL volume containing 0.1M phosphate buffer containing 100 μM DTPA, pH 7.4, at room temperature. The spectra were generated by the reaction of 100 mM DMPO, 33 mM HCO_3^-, 25 mM glucose, 7.5 U glucose oxidase and 1 μM b SOD1 with 0 mM PBZ and 0 mM INH (A), 0 μM PBZ and 4 mM INH (B), 4 mM PBZ and 0 mM INH (C), 4 mM PBZ and 4 mM INH (D). The omission of SOD (E) from reaction (D) showed no free radical detection in the spectrum.

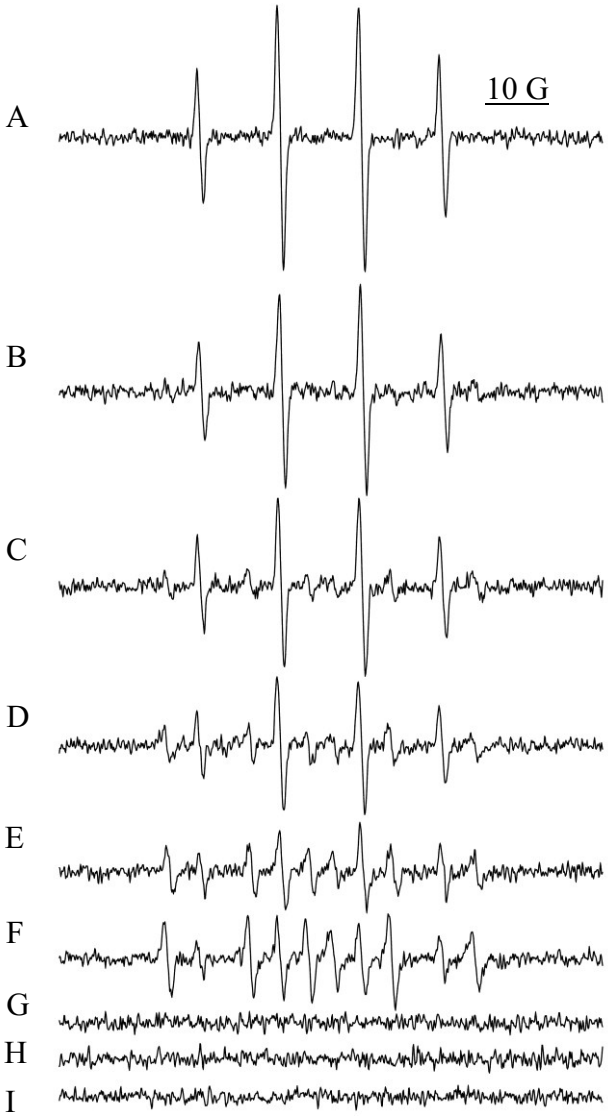

Figure A1.4 Concentration-dependent attenuation of the DMPO/$^{\bullet}$OH adduct with increasing sulfinpyrazone (SULF) concentration. All reactions were carried out using a 200 µL volume containing 0.1M phosphate buffer containing 100 µM DTPA, pH 7.4, at room temperature. The spectra were generated by the reaction of 100 mM DMPO, 33 mM HCO_3^-, 25 mM glucose, 7.5 U glucose oxidase and 1 µM SOD with 0 µM SULF (A), 250 µM SULF (B), 500 µM SULF (C), 1 mM SULF (D), 2 mM SULF (E), and 4 mM SULF (F). The omission of either SOD (G) or glucose/glucose oxidase (H) or HCO_3^-(I) from reaction (F) showed no free radical detection in the spectrum.

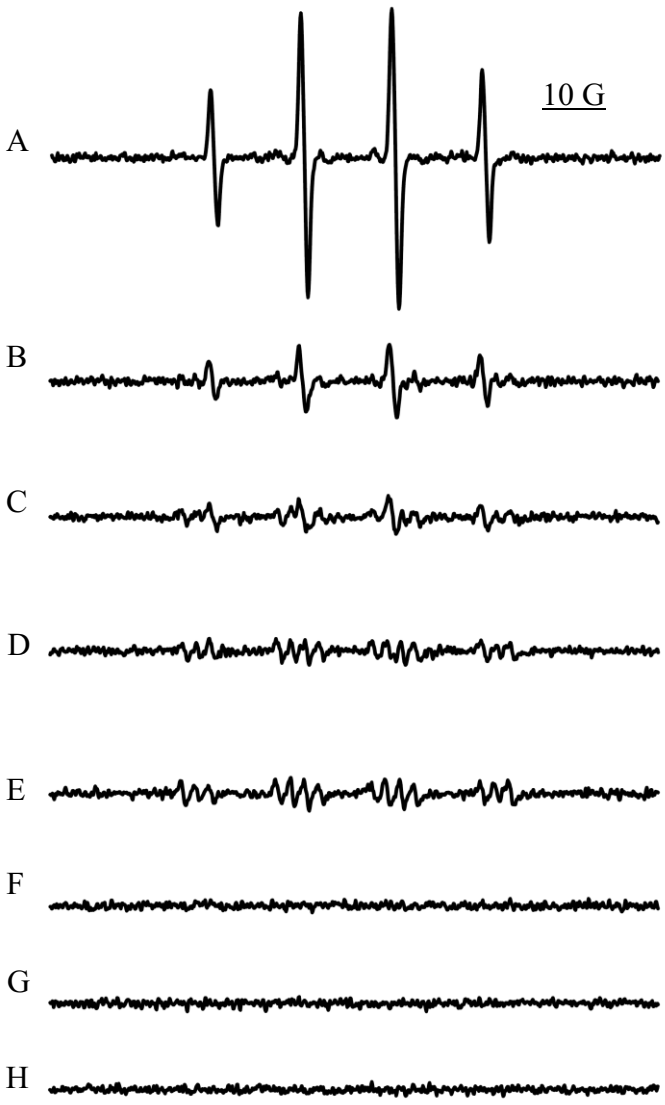

Figure A1.5 Concentration-dependent attenuation of the DMPO/$^{\bullet}$OH adduct with increasing aminoglutethimide (AG) concentration. All reactions were carried out using a 200 μL volume containing 0.1M phosphate buffer containing 100 μM DTPA, pH 7.4, at room temperature. The spectra were generated by the reaction of 100 mM DMPO, 33 mM HCO_3^-, 25 mM glucose, 7.5 U glucose oxidase and 1 μM SOD with 0 μM AG (A), 500 μM AG (B), 1 mM AG (C), 2 mM AG (D), and 4 mM AG (E).The omission of either SOD (F) or glucose/glucose oxidase (G) or HCO_3^-(H) from reaction (E) showed no free radical detection in the spectrum.

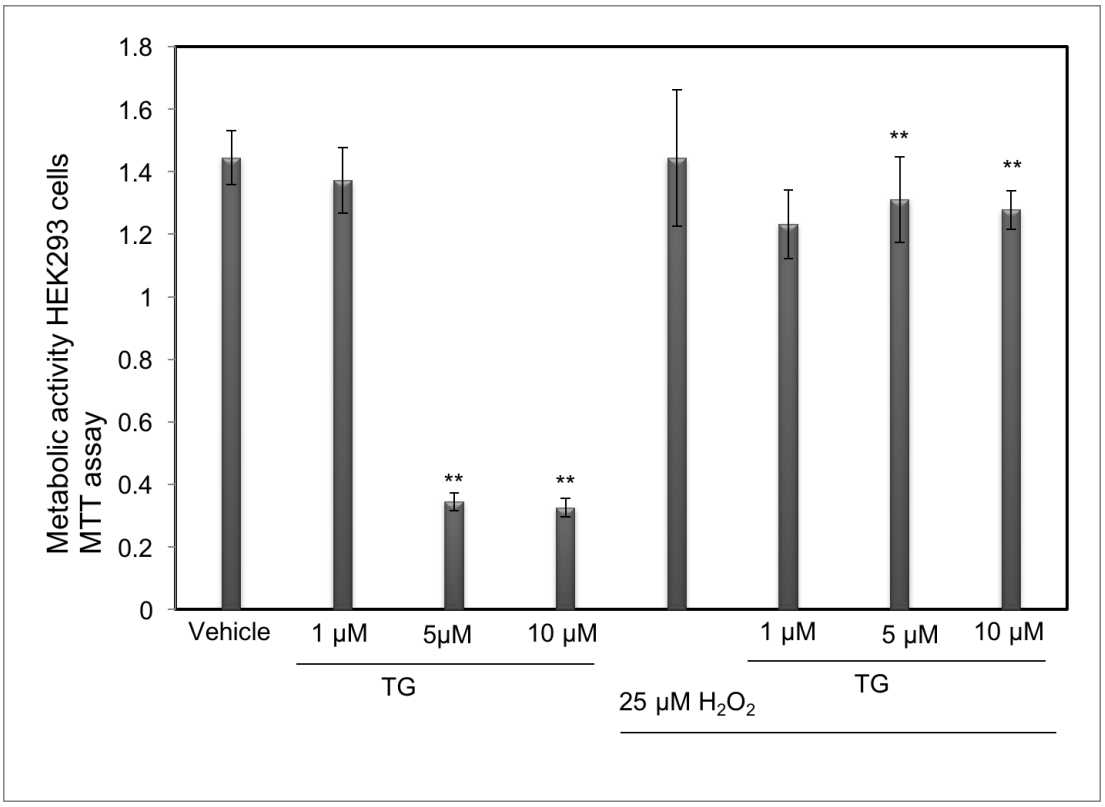

Figure A1.6 Effect of 6-TG, H_2O_2 and the combination of 6-TG and H_2O_2 on the metabolic activity of HEK293 cells. Cells were incubated in the presence of different concentrations of 6-TG (1, 5, 10 µM) either with the presence or absence of 25 µM H_2O_2 that was preincubated with HCO_3^- and drug for 60 min. prior to adding to cells. The metabolic activity of HEK293 cells was determined using tetrazolium–based assay (MTT) at 72 h. Values are the means ± SD (8 wells).

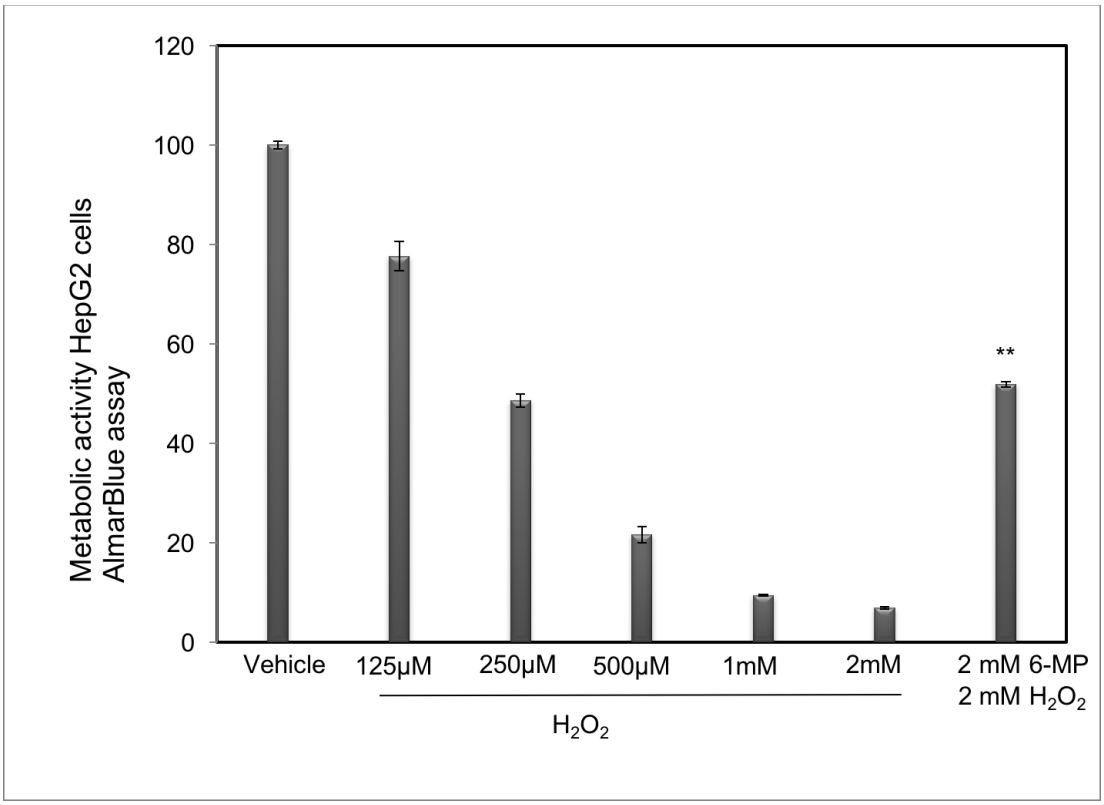

Figure A1.7 Effect of 6-MP on the cytotoxic effect of H_2O_2. Cells were incubated in the presence of different concentrations of H_2O_2 (125, 250, 500, 1000, 2000 µM) and in the presence of both 2 mM 6-MP and 2 mM H_2O_2 for 24 h. The metabolic activity of HepG2 was determined using alamarBlue assay. Values are the means ± SD (5 wells).

Appendix 2

List of publications and Long-term research interests

A2.1 List of publications

1. Michail K, **Aljuhani N,** Siraki AG. "The Interaction of Diamines and Polyamines with the Peroxidase-catalyzed Metabolism of Aromatic Amines – A Potential Mechanism for Modulation of Aniline Toxicity." Canadian Journal of Physiology and Pharmacology (2012) 91:228-35.

2. **Aljuhani N**, Michail K, Karapetyan Z, Siraki AG. "The effect of bicarbonate on menadione-induced redox cycling and cytotoxicity: potential involvement of the carbonate radical." Canadian Journal of Physiology and Pharmacology (2013) 91:783-90.

3. Michail K, Baghdasarian A, Khan MS, **Aljuhani N**, Narwaley M, Siraki AG. "Scavenging of Free-Radical Metabolites of Aniline Xenobiotics and Drugs by Amino Acid Derivatives: Toxicological Implications of Radical-Transfer Reactions." Chemical Research in Toxicology (2013) 26:1872-83.

4. **Aljuhani N**, Whittal RM, Khan SR, Siraki AG. "Phenylbutazone oxidation via SOD Peroxidase Activity: an EPR study." Chemical Research in Toxicology (2015) 28:1476-83.

5. Khan SR, Nagar PH, Fahlman R, Jurasz P1, Michail K, **Aljuhani N**, Siraki AG. "Proteomic profile of aminoglutethimide-induced apoptosis in HL-60 cells: Role of myeloperoxidase and arylamine free radicals." Chem Biol Interact. (2015) 239:129-138.

6. Khan SR, **Aljuhani N**, Morgan A,Baghdasarian A, Siraki AG. "Cytoprotective effect of isoniazid against H_2O_2 derived injury in HL-60 cells Chem Biol Interact. (2015).

7. Aldawsari FS, Aguiar RP, Wiirzler LA, Aguayo-Ortiz R**, Aljuhani N**, Cuman RK, Medina-Franco JL, Siraki AG, Velázquez-Martínez CA."Anti-inflammatory and antioxidant properties of a novel resveratrol-salicylate hybrid analog." Bioorg Med Chem Lett. 2016 Mar 1;26(5):1411-5.

8. Khan SR, Morgan AG, Michail K, Srivastava N, Whittal RM, **Aljuhani N**, Siraki AG."Metabolism of isoniazid by neutrophil myeloperoxidase leads to isoniazid-NAD(+) adduct formation: A comparison of the reactivity of isoniazid with its known human metabolites." Biochem Pharmacol. 2016 Apr 15;106:46-55.

9. **Aljuhani N**, Spryut L, Reiz B., Whittal RM, Siraki AG. "Potential involvement of SOD and peroxymonocarbonate in the oxidation of anticancer drug metabolite, 6-mercaptopurine." In processing for publication. (FRBM).

10. **Aljuhani N**, N.W.M. Commandeur, Khan SR, Siraki AG. "Role of SOD1 in phenylbutazone reactivity: Toxicological implications in human hepatoma cells (HepG2)." In processing for publication. (CRT).

A2.2 Long-term research interests

There is a demand for personalized approaches to combat the cancer disease, that is a major cause of death throughout the world, and to ameliorate the side effects of drugs that are used in treating deadly diseases. Thus, understanding the interplay between the drugs and peroxidase enzymes-derived oxidative stress including SOD1 and COX-2 will highlight the development of a promising approach to selectively killing cancers and thereby that will enhance the quality-of-life of human afflicted with these deadly diseases. Nevertheless, my long-term research interest (**at Taibah University**) will be:

- **Exploring the role of COX-2 peroxidase-derived oxidative stress in oxidizing drug and its cytotoxicity.**

- **Understanding the role of both SOD peroxidase activity and COX-2 peroxidase-generated oxidative stress in anti-cancer drugs resistance.**