DIDOX MODULATES REACTIVE OXYGEN SPECIES PRODUCTION AND INFLAMMATORY EVENTS INDUCED BY LIPOPOLYSACCHARIDE (LPS) AND ARYL HYDROCARBON RECEPTOR (AHR) LIGANDS IN RAW 264.7 MURINE MACROPHAGES.

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DIDOX MODULATES REACTIVE OXYGEN SPECIES PRODUCTION AND INFLAMMATORY EVENTS INDUCED BY LIPOPOLYSACCHARIDE (LPS) AND ARYL HYDROCARBON RECEPTOR (AHR) LIGANDS IN RAW 264.7 MURINE MACROPHAGES.

A dissertation presented to the Graduate School of Clemson University

In Partial fulfillment of the requirements for the Degree Doctor of Philosophy Biological Sciences

By

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Dr. Charles D. Rice, Committee Co-Chair
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ABSTRACT

Aberrant activation of macrophages during inflammation results in oxidative burst release of reactive oxygen and reactive nitrogen species (ROS/RNS) which are widely accepted to participate in pathogenesis of cancer, cardiovascular disease, atherosclerosis, hypertension, ischemia/reperfusion injury, diabetes mellitus, neurodegenerative diseases (Alzheimer’s disease and Parkinson’s disease), rheumatoid arthritis, and ageing. It is demonstrated here that Didox (3,4-Dihydroxybenzohydroxamic acid) possesses antioxidative and anti-inflammatory properties that can protect against inflammation and oxidative stress induced by lipopolysaccharide (LPS) and aryl hydrocarbon receptor (AHR) ligands (PCB126, E804, and IO) in Raw 264.7 murine macrophages. In brief, it is demonstrated here that Didox inhibits LPS-induced oxidative stress, ROS/RNS generations, IL-6 production, NO production, phagocytic activity, and NF-κB nuclear translocation in Raw 264.7 murine macrophages. Furthermore, Didox downregulated ROS production, cytochrome P450 1A1 (CYP1A1), and AHR nuclear translocation induced by PCB126, E804, and IO in Raw 264.7 murine macrophages. These data suggest that oxidative stress regulatory effects of Didox may be useful in managing inflammatory diseases conditions initiated by exposure to AHR activating xenobiotics or LPS.
DEDICATION

I dedicate this work to my name-sake, the late Ramogolo Thabe P Matsebatlela, who took every opportunity to encourage me in my educational and life endeavors; and in his final breath articulated and expressed his best wishes to my life-goals. I extend the dedication to Rangwane Mphalaborwa Matsebatlela and Malome Thomas Mahlo whose life-times expired during the process of completion of my studies and they did not live to witness successful conclusion of my project. May their souls rest in peace.
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CHAPTER 1: LITERATURE REVIEW

1.1 Inflammation and Oxidative stress

Inflammation is a complex stereotypical response of the damage to the body cells and vascularized tissues. The inflammatory response is phylogenetically and ontogenetically the oldest defense mechanism that is controlled by cytokines, products of the plasma enzyme systems, lipid mediators released from different cells, and by vasoactive mediators released from mast cells, basophils, platelets and macrophages (Ross et al., 2002). Inflammation is the complex immunological response of vascular tissues to exogenous or endogenous harmful stimuli, such as pathogens, damaged cells, or irritants. It is a protective attempt by the immune system to remove the injurious agents as well as initiate the healing process for tissues.

When inflammation is lacking, wounds and infections persist and never heal while progressive destruction of body tissue continue to compromise the survival of the organism. However, aberrant inflammation can also lead to a host of diseases, such as atherosclerosis, cancer, respiratory syndromes, multiple organ dysfunction and rheumatoid arthritis (Fig 1.1, below). It is for that reason that inflammation is normally closely regulated by the body. Inflammation can be classified as either acute or chronic according to duration of response (Khansari et al., 2009).
Acute inflammation is the relatively shorter initial response of the body to harmful stimuli and is achieved by the increased movement of plasma and leukocytes from the blood into the site of injury or infection. It is a short-term process, usually appearing in a few minutes or hours and ceasing once the injurious stimulus has been removed. It is characterized by five cardinal signs: rubor (redness), calor (increased heat), tumor (swelling), dolor (pain), and functio laesa (loss of function). Recruitment of leukocytes and production of various mediators trigger a cascade of biochemical events which propagates and matures the inflammatory response, involving the local vascular system, the immune system, and various cells within the injured tissue. Prolonged inflammation, known as chronic inflammation, leads to a progressive shift in the type of cells which are present at the site of inflammation and is characterised by a lengthy simultaneous destruction and healing of the tissue from the inflammatory process (Ferrero-Miliani et al., 2007).

Chronic inflammation is a pathological condition characterized by continued active inflammation response and tissue destruction. Many of the immune cells including macrophages, neutrophils and eosinophils are involved directly or by production of inflammatory cytokines in pathology of chronic inflammation. Chronic inflammation can be a major cause of cancers and neurodenegerative diseases. Moreover, many studies suggest that chronic inflammation could play a serious role in wide variety of age-related diseases including diabetes, cardiovascular and autoimmune diseases. Inflammatory processes induce oxidative stress and reduce cellular antioxidant capacity (Khansari et al., 2009).
Figure 1.1 Consequences of aberrant inflammation and oxidative stress in physiology. Oxidative stress and chronic inflammation can lead to a wide variety of disease conditions in multiple organs, rendering the usually protective immune reaction a liability rather than an asset.

Overproduced free radicals react with cell membrane fatty acids and proteins impairing their function permanently. In addition, free radicals can lead to mutation and DNA damage that can be a predisposing factor for cancer and age-related disorders (Khansari et al., 2009).
The generation of reactive oxygen species (ROS) and other free radicals during metabolism is a necessary and normal process that ideally is compensated for by an elaborate endogenous antioxidant system. However, due to many environmental, lifestyle, and pathological situations, excess radicals can accumulate, resulting in oxidative stress (Figure 2). Oxidative stress has been related to cardiovascular disease, cancer, and other chronic diseases that account for a major portion of deaths today. Antioxidants are compounds that hinder the oxidative processes and thereby delay or prevent oxidative stress (Khansari et al., 2009).

1.1 (a) Reactive Oxygen intermediates and oxidative stress

Reactive oxygen species (ROS) are a cluster of highly unstable compounds that are generated during inflammation and normal cellular metabolism via incomplete reduction of molecular oxygen. They consist of molecules with unpaired electrons such as superoxide anions (O2^-) and hydroxyl radical (•OH), and molecules that have oxidizing propensity but do not possess free electrons, for example hydrogen peroxide (H2O2) and hypochlorous acid (HOCl) (Abid et al., 2005). These species are involved in the regulation of fundamental cellular activities such as cell growth and differentiation (Dor and Porat, 2001; D'Angio and Maniscalco, 2002, Hopf et al., 2005, and Veal et al., 2007). However, their overproduction and/or the presence of impaired antioxidant ability results in oxidative stress which can induce and propagate significant injury (Fig 1.2).
Likewise, reactive nitrogen species (RNS), the by-products of nitric oxide (NO) metabolism, play an important role in maintaining various physiological functions at basal levels, and contribute to several pathological processes at high levels. Examples of RNS include nitrite (NO$_2^-$), nitrate (NO$_3^-$) and peroxynitrite (ONOO$^-$).

Figure 1.2: Antioxidants and reactive oxygen species (ROS) exist in physiological balance and are required for normal cellular function. Excessive generation of free radicals generation of free radicals such as OH, NO, O$_2^-$, H$_2$O$_2$ and deficiency of antioxidants can lead to oxidative stress whereas the reverse leads to ROS deficiency.
As these reactive oxygen and nitrogen species are deemed as second messengers, it is important to note that they should have four basic characteristics that allow regulation. (1) They are either enzymatically generated or regulated by channels and pumps (2) They are enzymatically degraded. (3) Their concentration rises and falls within a short period, and (4) They can be specific in action (Boueiz and Hassoun, 2009).

1.1 (b) Reactive oxygen species

As divalent reduction of $O_2$ to form $H_2O_2$ is spin restricted, one electron reduction is favored and $O_2^-$ is readily formed. Therefore, the major source of $H_2O_2$ in cells arises from the dismutation of $O_2^-$. At physiological pH, non-enzymatic dismutation is rapid, but dismutation is markedly accelerated by the superoxide dismutases (SOD) to near the limit of diffusion. $H_2O_2$ can diffuse readily through membranes, probably through the same channels as water. $H_2O_2$ is not a radical and does not spontaneously react with carbon-centered molecules or thiols. It does, however, react rapidly with thiolates (–$S^-$) and transition metals. Hydroxyl radical ($OH^\cdot$) can be generated when $H_2O_2$ collides with a reduced transition metal, such as ferrous iron ($Fe^{2+}+H_2O_2\rightarrow Fe^{3+}+OH^-+OH^\cdot$) in a Fenton reaction. The distribution of transition metals in the cell is generally well regulated and so-called free transition metals are associated with pathology rather than signaling. The hydroxyl radical is so reactive that it will generally abstract a hydrogen from the closest molecule, producing water and another radical. As damage to the
molecule would probably ensue, it seems rather unlikely that OH’ could act as a second messenger. On the contrary, O$_2$’$^-$ and H$_2$O$_2$ have all four of the characteristics of second messengers as defined above, i.e. they are generated enzymatically, degraded by SOD or catalase and glutathione peroxidases, are present at very low concentrations but can transiently be elevated to measurable amounts and both react only at specific sites, such as metals and thiolates (Khansari et al., 2009).

1.1 (c) Reactive nitrogen species

The NO molecule itself has little chemical reactivity with organic molecules and primarily binds to metals as in the regulatory site of guanylate cyclase and in hemoglobin. In fact, guanylate cyclase has been shown to require NO binding to its regulatory site in order to be active. Moreover, NO interacts with O$_2$’$^-$ to form ONOO$^-$, a reaction that is extremely fast ($6.7 \times 10^9$ mol$^{-1}$ s$^{-1}$) and is determined by the diffusion rate of NO, the relative amount of each radical, and their ability to react with oxyHb for NO and SOD for O$_2$’$^-$. Thus, this reaction can only occur in localized areas close to the source of O$_2$’$^-$ release (Wink and Mitchell, 1998). The peroxynitrite molecule is not itself highly reactive; however, its acid form, peroxynitrous acid (ONOOH) is very unstable and reactive, having properties similar to OH’ and nitrogen dioxide (NO$_2$). The formation of peroxynitrous acid is thought to be the mechanism through which NO production can lead to toxicity and the predominant mechanism of O$_2$’$^-$-mediated toxicity when Fenton chemistry
is not involved. It was widely assumed that ONOO\textsuperscript{−} was destroyed through non-enzymatic degradation until recent studies suggested that glutathione peroxidase could catalyze the reduction of ONOO\textsuperscript{−} to NO\textsubscript{2}\textsuperscript{−} (Sies and Arteel, 2000). That an enzymatic mechanism appears to exist for its elimination is consistent with a role for ONOO\textsuperscript{−} as a second messenger. Peroxynitrite can interact with the cysteine residues of proteins or glutathione, resulting in formation of S-nitrosothiols and providing both a mechanism for regulation of thiol-dependent enzymatic activities and a source of bioavailable NO that can be exchanged between thiols. Peroxynitrite also modifies tyrosine residues, producing 3-nitrotyrosine (3NT). While thought to be an irreversible process associated with injury, tyrosine nitration may have a signaling role and be enzymatically reversed. Perhaps like H\textsubscript{2}O\textsubscript{2}, ONOO\textsuperscript{−} participates in signaling processes at low concentrations but, at greater concentrations, produces toxicity (Boueiz and Hassoun, 2009).

1.1 (d) Antioxidant defense and redox signaling

The production of reactive oxygen and nitrogen species is a steady-state event in respiring cells, and uncontrolled production of these reactive species is the primary cause of some major degenerative conditions. Cellular defense, targeted against these transient but damaging species, can be grouped under several mechanisms, and collectively they operate to terminate free radical reactions or remove reactive species and their secondary products. Since oxygen and nitrogen atoms of reactive oxygen intermediates (ROI) contain unpaired
electrons in their outer electron shells, they can react with other molecules to restore their electron pairing. These could lead to damaging of biological molecules such as nucleic acids, proteins, and lipids. To minimize heavy metal–induced catalysis of the Fenton and Haber-Weiss reactions that generate the most reactive hydroxyl radical, expression of several specific metal-binding proteins such as haptoglobin, ceruloplasmin, ferritin, transferrin, lactoferrin, and albumin, ensures that these metals remain cryptic, thereby serving as a key preventive measure in the overall free radical defense system. Antioxidant enzymes, most of which are highly dependent upon exogenous co-factors for their activity, can dismutate superoxide anion (superoxide dismutase) directly into hydrogen peroxide which in turn can be removed by catalase or several forms of glutathione peroxidase.

Finally, direct quenching of radicals can be achieved by a series of endogenous and exogenous antioxidants. Functioning as scavengers, these molecules donate electrons, and themselves become free radicals that can either initiate chain reactions or conversely, be regenerated. It is now known that active repair pathways exist in which endogenous antioxidants such as glutathione, ubiquinol and NADH/NADPH, are obligatory in the recycling pathways via redox reactions. It has long been known that control of the intracellular redox environment is vital for proper cellular function. To protect themselves from the constant oxidative challenge, cells have developed defense mechanisms that insure the proper balance between the prooxidant and antioxidant molecules. In addition to the enzymes that remove ROS, such as SOD, catalase and glutathione peroxidase,
glutathione (GSH/GSSG) and thioredoxin (Trx) systems are key players in the maintenance of the redox status of the cells, defined as the ratio of the concentration of oxidizing equivalents to that of reducing equivalents. Members of the Trx family have a conserved catalytic site with two cysteine residues that undergo reversible oxidation to form a disulfide bridge through the transferring of reducing equivalent to a disulfide substrate. The Trx then reverts to the reduced form through the action of Trx reductase, an NADPH flavoprotein. Similarly, the GSH/GSSG ratio is controlled by glutathione reductase. Peroxiredoxins are another class of enzymes that can reduce \( \text{H}_2\text{O}_2 \) using thiols (Rhee et al., 1999), some using thioredoxin as a co-substrate and at least one using glutathione (Chen et al., 2000).

The antioxidant enzymes play a significant role in protection against cell injury. Nonetheless, these enzymes may also play an additional role in signaling by counterbalancing the production of ROS/RNS. Indeed, one may think of a role for glutathione peroxidase in decreasing \( \text{H}_2\text{O}_2 \) concentration as analogous to the role of phosphodiesterase in decreasing cAMP concentration. In other words, the antioxidant enzymes provide the turn-off component of redox signaling. The interplay between ROS signaling and inflammation involves a complex cellular network comprising vascular cells and inflammatory and immune cells. Among them, macrophages have a unique central role and mediator of co-ordinated cellular and intercellular inflammatory reactions.
1.1 (e) Oxidative stress and NF-κB signaling

The nuclear factor associated κB (NF-κB) transcription factor plays a major role in coordinating innate and adaptative immunity, cellular proliferation, apoptosis and development. Since the discovery in 1991 that NF-κB may be activated by H₂O₂, several laboratories have put a considerable effort into dissecting the molecular mechanisms underlying this activation. Whereas early studies revealed an uncharacteristic mechanism of activation, leading to IκBα phosphorylation independently of IκB kinase (IKK), recent findings suggest that H₂O₂ activates NF-κB mainly through the classical IKK-dependent pathway. The molecular mechanisms leading to IKK activation are, however, cell-type specific and the description of the effect of other ROS and RNS on NF-κB activation comes essential. Recent data also highlights the role of ROS in NF-κB activation by proinflammatory cytokines (TNF-α and IL-1β) and lipopolysaccharide (LPS), two major components of macrophages-mediated innate immunity (Gloire et al., 2006).

The transcription factor NF-κB is crucial in a series of cellular processes, such as inflammation, immunity, cell proliferation and apoptosis. It consists of homo- or heterodimers of a group of five proteins, namely NF-κB1 (p50 and its precursor p105), NF-κB2 (p52 and its precursor p100), p65/RelA, c-Rel and RelB. In the resting state, NF-κB is sequestered in the cytoplasm of the cell through its tight association with inhibitory proteins called IκBs, comprising IκBα, IκBβ, IκBγ, IκBε, Bcl-3, p100 and p105. Upon cell stimulation, IκB proteins are rapidly
phosphorylated and degraded by the proteasome, and the freed NF-κB translocates into the nucleus to regulate the expression of multiple target genes (Hayden and Ghosh, 2004).

The classical NF-κB-activating pathway is induced by a variety of mediators, such as pro-inflammatory cytokines (TNFα, IL-1β) and LPS binding to Toll-like receptors (mainly TLR4). Although these NF-κB inducers signal through different receptors and adaptor proteins, they all converge to the activation of IκB-kinase (IKK) complex, which includes the scaffold protein NF-κB essential modulator (NEMO, also called IKKγ), IKKα and IKKβ kinases. Once activated by phosphorylation, the IKK complex phosphorylates IκBα on Ser32 and Ser36, which is subsequently ubiquitinated and degraded via the proteasome pathway. The freed NF-κB then translocates into the nucleus where it activates the transcription of target genes such as cytokines (e.g. IL-6), chemokines, adhesion molecules, inflammatory enzymes (e.g. iNOS) and apoptosis mediators (Bonizzi and Karin, 2004).

Beside this classical activation, a novel NEMO-independent NF-κB-activating pathway, important for secondary lymphoid organ development and homeostasis and adaptive immunity, was described. It is induced by B-cell activating factor (BAFF), lymphotoxin β (LTβ), CD40 ligand and human T cell leukemia (HTLV) and Epstein-Barr (EBV) virus. The NEMO-independent NF-κB-activating pathway enhances NF-κB inducing kinase (NIK)- and IKKα-dependent
processing of p100 into p52, which binds DNA in association with its partners, like RelB. These stimuli also activate the classical pathway (Gloire et al., 2006).

In 1991, Schreck et al. were the first to demonstrate that direct addition of H$_2$O$_2$ to the culture medium of a subclone of Jurkat cells (Jurkat JR) could activate NF-κB (Schreck et al., 1991). Since this discovery, several laboratories have put considerable efforts into dissecting the molecular mechanisms underlying this activation. The results that came out of these studies suggest that NF-κB activation by H$_2$O$_2$ is highly cell-type specific and involves quite different mechanisms, making the role of H$_2$O$_2$ in macrophage signaling a subject of great importance since they produce it in large amounts during inflammatory reactions. Although the vast majority of studies concerning oxidant-induced NF-κB activation have focussed on H$_2$O$_2$, other oxidants, like hypochlorous acid (HOCl) and singlet oxygen (‘O$_2$), have been shown to modulate NF-κB activation. On the other hand, some works have also highlighted NF-κB regulation by peroxinitrite which is a reactive nitrogen species (Gloire et al., 2006).

To explain the diversity of inducers that activate NF-κB via the same IKK-dependent pathway, a model has emerged suggesting that all NF-κB activators cause an oxidative stress that is mainly responsible for IKK activation and IκBα degradation. This model is based on several observations, including that most of
NF-κB-inducers trigger the formation of ROS (Los et al., 1995) and that several antioxidants can block NF-κB activation (Schreck et al., 1992). As a result of research in these areas, the effects of different antioxidants (NAC, PDTC and GSH) or over-expression of antioxidant enzymes (SOD) on NF-κB activation by TNFα and IL-1β or LPS has been well documented. It will therefore be very interesting to find out what effect ROS and RNS will have on activated macrophages in searching for agents that can modulate macrophage activity.

1.2 Macrophages

Macrophages are key players in inflammation and their wide tissue distribution makes them well suited to provide an immediate defense against foreign elements prior to leukocyte migration (Zhang et al., 2008). Macrophages have long been considered to be important immune effector cells. Élie Metchnikoff, who won the Nobel Prize 100 years ago for his description of phagocytosis, proposed that the key to immunity was to "stimulate the phagocytes" (Nathan, 2008). Since this discovery, immunologists have been occupied with the concept of macrophages as immune effector cells and with understanding how these cells participate in host defense. Macrophages are present in virtually all tissues. They differentiate from circulating peripheral-blood mononuclear cells (PBMCs), which migrate into tissue in the steady state or in response to inflammation. These PBMCs develop from a common myeloid progenitor cell in the bone marrow that is the precursor of many different cell types, including neutrophils, eosinophils,
basophils, macrophages, dendritic cells (DCs) and mast cells. During monocyte development, myeloid progenitor cells (termed granulocyte/macrophage colony-forming units) sequentially give rise to monoblasts, pro-monocytes and finally monocytes, which are released from the bone marrow into the bloodstream.

Figure 1.3: Macrophages play a central in inflammation by interacting with other inflammatory cells. Macrophages interact with neutrophils (NΦ), dendritic cells (DC), B cells and T cells (Th1/h2) to co-ordinate a successful immune attack in attempt to remove the injurious stimuli. During chronic inflammation, macrophages release a plethora of mediators which not only lead to direct tissue injury but also recruit far too many inflammatory cells for prolonged period to a site of injury.
Monocytes migrate from the blood into tissue to replenish long-lived tissue-specific macrophages of the bone (osteoclasts), alveoli, central nervous system (microglial cells), connective tissue (histiocytes), gastrointestinal tract, liver (Kupffer cells), spleen and peritoneum.

Macrophages play a pivotal role in innate and adaptive immunity by interacting with other immunological and non-immunological cells to insure success of the inflammatory process and clearance of invaders. As such, they play a central role by interacting with cells such as neutrophils, dendritic cells, T cells, B cells, Natural killer cells and fibroblasts (Fig 1.3). Concerning macrophage activation and phagocytosis, large volumes of macrophage research have unraveled the induction of cytokines such as IL-1β, IL-6, IL-10, TNF-α and transforming growth factor (TGF); chemokines such as monocyte chemoattractant protein-1 (MCP-1) and macrophage inflammatory protein-2 (MIP-2); the reactive oxygen species (ROS), reactive nitrogen species (RNS) and nitric oxide (NO)-generated mainly through iNOS.

During microbial contact, many parallel signaling pathways are simultaneously activated that together define the phagocyte response and regulate internalization of microbes by macrophages. Phagocytosis is a complex system with special emphasis on four sources of that complexity. First, many different receptors recognize microbes, and phagocytosis is usually mediated simultaneously by multiple receptors. Second, different microbe-recognition
receptors induce different signaling pathways, and these signals interact cooperatively (and sometimes destructively) to mediate ultimate responses to particles. Third, microbe recognition is coupled (either directly through phagocytic receptors or indirectly through co-receptors) to inflammatory responses that in turn affect the efficiency of particle internalization by the phagocyte or neighboring phagocytes. Fourth, many pathogenic microbes actively attempt to regulate the mechanisms of phagocytosis to evade destruction. In this review we have focused our discussion specifically on phagocytosis of microbes.

Phagocytosis also is required for normal clearance of apoptotic cells, a process in which many of the same levels of complexity apply (Underhill and Ozinsky, 2002).

Particle internalization by macrophages is accompanied by activation of many signaling pathways that together orchestrate rearrangement of the actin cytoskeleton, extension of the plasma membrane, and engulfment. Dozens of signaling molecules including actin binding proteins, membrane traffic regulators, ion channels, kinases, and lipases are activated during phagocytosis of complex particles (such as opsonized bacteria) and may contribute to efficient internalization. Linking these proteins in a model of phagocytosis is a challenge, and identifying the key regulators is a task that requires a concerted effort. However, certain signaling molecules stand out both as participating in phagocytosis and as participating in many other signaling pathways. Phosphoinositide 3-kinase (PI 3-kinase), phospholipase C (PLC), Rho GTPases,
and PKC are integration points for regulating of phagocytosis. Furthermore, these molecules not only orchestrate the mechanics of particle ingestion, they also regulate inflammatory responses and microbial killing (Underhill and Ozinsky, 2002).

PI 3-kinase catalyzes phosphorylation of PI(4,5)P$_2$ to PI(3,4,5)P$_3$, a phospholipid important in recruiting signaling molecules such as the kinase AKT/PKB to specific regions of membranes. Pharmacological inhibition of PI 3-kinase blocks phagocytosis of IgG- and complement-opsonized particles, unopsonized zymosan, and bacteria, although the requirement may not be absolute because the uptake of IgG-opsonized particles smaller than 3 microns across is less affected than the uptake of larger. Inhibition of PI 3-kinase blocks membrane extension and fusion behind the bound particle, perhaps due to a failure to insert new membrane at the site of particle internalization (Cox et al., 1999).

In recent work using cells from knockout mice and microinjection of inhibitory antibodies, Vieira and colleagues have demonstrated that these stages of phagocytosis require class I PI 3-kinase, while a second type of PI 3-kinase, class II/VPS34, is required for phagosome maturation (Vieira et al., 2001).
Phosphoinositiode-specific phospholipase C (PI-PLC) mediates cleavage of PI (4, 5)P$_2$ resulting in release of IP$_3$ and DAG, second messengers that mobilize intracellular Ca$^{++}$ stores and activate protein kinase C family members, respectively. PI-PLC is recruited to phagosomes containing IgG-opsonized particles, and inhibition of its activity blocks particle internalization. Like protein kinase C inhibitors, PLC inhibitors completely block the formation of actin filaments beneath the site of particle contact, suggesting that the main role of PLC in particle internalization is to activate protein kinase C (Botelho et al., 2000). In addition to being required for the mechanical aspects of particle internalization, PI 3-kinase and PI-PLC also are implicated in pro-inflammatory signaling induced by particulate stimuli. Thus, PI 3-kinase is recruited to Toll-like receptors when cells are stimulated with heat-killed S. aureus, and activation of PI 3-kinase has been implicated in NF-κB-mediated cytokine production. Similarly, PI-PLC activity is required for microbe-induced pro-inflammatory signaling in macrophages primarily due to its role in activating PKC (Chen et al., 2000).

Macrophages utilize phagocytic receptors and other pattern recognition receptors, such as TLRs, to identify microbes and trigger immune defenses. When microbes are engulfed by macrophages, TLR pathways activate the IL-1β and inflammatory cytokine promoter, and the full repertoire of immune responses is activated, and the IL-6 promoter and an NF-κB reporter are induced. Indeed,
TLR pathways are vigorously activated by mycobacterial exposure, but somehow some microbes are able to survive the consequences of the wide spectrum of cytokines produced. One important mechanism of microbial killing is the production of reactive nitrogen species activated by a variety of microbial components through TLR pathways. TLR-mediated induction of calcium-independent nitric oxide synthase (iNOS) is necessary for killing of some microbes. In human macrophages, TLR2 activates other potent microbicidal mechanisms that are able to kill mycobacteria (Thoma-Uszynski et al., 2001). Microbes may induce iNOS through TLR2/TLR4/MyD88-independent mechanisms, suggesting the involvement of receptors other than TLRs. Such cross-talk between TLRs and other innate immune recognition receptors has long been recognized in killing caused by production of reactive oxygen species, where LPS primes activation of phagocyte oxidase induced by phorbol ester or by phagocytic particles that do not utilize TLR pathways (Pabst and Johnston, 1980).

The process of phagocytosis also requires the recruitment of actin filaments for the internalization process. This actin recruitment by polymerization/depolymerization exhibits the critical step during the phagocytic process. Cytochalasin-D (Cyt-D), known to block actin polymerization by binding to the barbed (plus) end of the actin filament, is described as a potent inhibitor of phagocytosis (Wakatsuki et al., 2001; Tjelle et al., 2000). During internalization,
activated macrophages generate ROS/RNS under activation of NADPH oxidase (Park, 2003). This ROS generation may also act as a second messenger, which can trigger several signalling pathways including the activation of NF-κB resulting in the production of pro-inflammatory cytokines such as IL-6 and TNF-α (Iles and Forman, 2002).

The discussion provided here demonstrates that phagocytosis is an inherently complex process that requires coordinated activation of signaling leading to events as diverse as actin remodeling, alterations in membrane trafficking, particle engulfment, microbial killing, and production of appropriate inflammatory mediators that direct the adaptive immune response. The consequences of phagocytosis vary, and they depend on the identity of the microbial target and the many factors that modulate the activation state of the phagocyte; for instance, phagocytosis can induce superoxide formation and apoptosis in macrophages (Park, 2003).

Many proteins have been identified that play important roles during phagocytosis, and the application of high-throughput technologies is accelerating their discovery. A central challenge in anti-inflammatory research will be to integrate these molecules into pathways and networks that account for the diversity of phagocytic responses. Pathogenic microorganisms have sought out vulnerabilities in phagocytic mechanisms in their attempts to subvert defenses, and the use of overlapping and redundant mechanisms during phagocytosis may
reflect evolutionary pressures for the development of a complex, though highly robust system. Studying of these networks and delineation of the phagocytosis process is important in defining molecular targets and candidates that can regulate macrophage phagocytic activity during inflammation.

1.3 Aryl hydrocarbon receptor (AHR) activation and oxidative stress

Increasing presence of environmental contaminants and poisonous polycyclic aromatic hydrocarbons leads to high incidence rate of contact with these toxicants and produces oxidative stress which raises a number of toxic and carcinogenic responses in experimental animals and humans mediated for the most part by the aryl hydrocarbon receptor (AHR) Barouki et al., 2007). The AHR is a ligand-activated transcription factor whose central role in the induction of drug-metabolizing enzymes has been well established and widely demonstrated.

It has been widely shown that the AHR molecule also functions in pathways outside of its role in detoxification and that modulation of these pathways by environmental AHR ligands may be an important part of the toxicity of these compounds. The AHR molecule is a cytosolic ligand-activated transcription factor that mediates many toxic and carcinogenic effects in animals and possibly in humans (Pollenz, 2002). Activation of the AHR pathway in vertebrates causes the toxic and carcinogenic effects of a wide variety of environmental contaminants such as dioxin (TCDD, 2,3,7,8-tetrachlorodibenzo-p-dioxin), coplanar polychlorinated biphenyls (PCBs) and polycyclic or halogenated
aromatic hydrocarbons (PAHs or HAHs). As a consequence of AHR activation, many detoxification genes are transcriptionally induced, including those coding for the Phase I xenobiotic-metabolizing cytochrome P450 enzymes of which CYP1A1 is one the central players. Other molecules discovered to possess ability to bind AHR include indirubin and indirubin-derivatives (Kawanishi et al., 2003).

Indirubin derivatives such as Indirubin-3’-(2,3 dihydroxypropyl)-oximether (E804) have been demonstrated to block Stat3 signaling in human breast and prostate cancer cells via interactions with AHR. Studies on AHR activation using indirubin derivatives demonstrated that although derivatives such 6-Bromoindirubin-3’-monoxime (BIO) and 5-Iodo-Indirubin-3’-monoxime (5-Iodo) bind to AHR and induce its nuclear translocation, they also triggered ARNT-independent nuclear redistribution of AHR (Knockaert et al., 2004). Indirubin-3-monoxime is an aryl hydrocarbon receptor (AHR)-binding, idole-derived compound known to be a potent inducer of CYP1A1, at least in hepatic cell lines and indirubin-3’-monoxime has been demonstrated to either act as classical AHR ligand, or modulates endogenous AHR-regulated activity in U937 histiocytic lymphoma cells (Springs and Rice, 2006).

The persistence and prevalence of various exogenous AHR ligands in environmental resources necessitates a search for molecules that can inhibit AHR-mediated oxidative stress in an attempt to ameliorate AHR-induced disease conditions.
1.4 Didox as a candidate for regulation of oxidative stress

Didox (3,4-Dihydroxybenzohydroxamine) is a powerful synthetic antioxidant and one of the most potent ribonucleotide reductase (RR) inhibitors, revealing an antitumor effect in several experimental studies and has been shown to have the capacity to downregulate NF-κB activation (Elford et al., 2007, Turchan et al., 2003, and Inayat et al., 2002). Several lines of research have demonstrated that Didox belongs to a unique class of more specific and more potent inhibitors of RR enzyme activity than hydroxycarbamides. Furthermore, Didox has has been shown to exhibit anti-tumour activity in several tumor models in both in vitro studies and in vivo models (Elford et al., 1979; Mayhew et al., 1997, 2002; Horvath et al., 2004a,b; Sumpter et al., 2004; Wakisaka et al., 2005).

Phase I trials, as well as phase II clinical studies, in patients with advanced breast cancer showed minimal toxicity (Veale et al., 1988; Carmichael et al., 1990; Rubens et al., 1991). It is therefore well established that Didox can be tolerated by patients at high dosage without major side effect; this makes Didox safe for use in clinical applications. Ribonucleotide reductase is a multienzyme responsible for the reduction of ribonucleotides to their corresponding deoxyribonucleotides, which are building blocks for DNA replication and repair in all living cells. The key role of RR in DNA synthesis and cell growth control has made it an important target for anticancer therapy. Increased RR activity has been associated with malignant transformation, tumor cell growth and viral replication. Based on the critical importance of RR in DNA synthesis and
potential as a point of DNA synthesis regulation, efforts for new RR inhibitors have been made in basic and translational research and Didox has been in the forefront.

Didox has been demonstrated to inhibit oxidative stress and secretion of inflammation mediators via suppression of NF-κB activity in various experimental models. Didox ability to inhibit this preeminent inflammatory transcription factor, NF-κB, is most probably due to its selective inhibition of phosphorylation of the cytoplasmic inhibitor IκBα (Lee et al., 2007). In addition to comprising a new class of antioxidants, Didox has been shown to be a potent chelator of iron, and free radical scavengers (Ding et al., 1992, Elford et al., 1989). Whether these interesting properties of Didox have other molecular effects that can be potentially therapeutic in the treatment of chronic inflammatory diseases is unknown. Didox, for example, protects against reperfusion injury in the rabbit heart (Ding et al., 1992). These interesting properties have led us to investigate if Didox has other molecular characteristics that are potentially therapeutic, especially in disease conditions mediated by deregulated macrophage activation.

Many cancers arise from sites of infection, chronic irritation and inflammation, making inflammatory cells (mainly macrophages) an important constituent of the tumor microenvironment. The ability of Didox to suppress inflammation and the important role of RR in DNA synthesis /repair makes it a worthy candidate and an active area of interest for anticancer, antivirus and anti-inflammation research.
Figure 1.4: A catalog of Didox properties that potentiates them as prolific candidates for treatments of inflammatory diseases. Although it was primarily synthesized as an inhibitor of ribonucleotide reductase, Didox has been shown to possess a variety of anti-cancer and anti-inflammatory characteristics.

Among other characteristics (Fig 1.4) Didox also mediates radiosensitizing effects in prostate cancer cells by abrogating the radiation-induced upregulation of Bcl-2 expression and NF-κB activity. The properties of Didox that make it a worthy anticancer candidate is well-demonstrated in its abilities to interfere with
the “Hallmarks of cancer” which explain how cancers acquire their survival capabilities. The introduction of adjacent hydroxyl groups increased inhibition of RR, and the presence of the hydroxyl-groups in Didox compounds made it active against L1210 leukemia. The presence of hydroxyl groups in the structure of Didox has been attributed to most its promising therapeutic potential.

Didox has been demonstrated to significantly reduce oxidative stress that was present in brain and cerebrospinal fluid (CSF) of HIV-infected patients. HIV-infected patients with dementia exhibit an accumulation of toxic substances in the CSF that are capable of inducing oxidative stress. The anti-inflammatory effect observed during treatment of HIV-infected patients using Didox presented it as novel compound capable of blocking the CSF-induced toxicity, the therapeutic potential of which is worthy of further exploration (Turchan et al., 2003). The oxidative stress inhibitory potential of Didox and its ability to attenuate NF-κB activity makes it a worthy candidate as a molecule that can modulate macrophage activity and its role in inflammation.

As therapeutic choices for the treatment of inflammatory diseases that are commonly chronic are coupled with severe side effects, the search for less harmful and effective compounds continues to be a strong research area (McKellar et al., 2007).
CHAPTER 2:  
DIDOX INHIBITS REACTIVE OXYGEN SPECIES PRODUCTION, INFLAMMATORY EVENTS, PHAGOCYTOSIS ACTIVITY AND NF-κB NUCLEAR TRANSLOCATION IN LPS-ACTIVATED RAW 264.7 MURINE MACROPHAGES.

ABSTRACT
Didox (3,4-Dihydroxybenzohydroxamic acid) is a synthetic antioxidant and one of the most potent ribonucleotide reductase inhibitors, revealing an antitumor effect in several experimental studies. In humans, tumorigenesis and many other diseases which include atherosclerosis, Parkinson’s disease, heart failure, myocardial infarction, Alzheimer’s disease, and multiple sclerosis have been strongly associated with elevated generation of inflammation and oxidative stress by aberrant activation of macrophages. The role of Didox on oxidative stress events produced in activated macrophages has not yet been fully investigated. The first objective was to examine the cytotoxic and cytostatic effect of Didox on Raw 264.7 cells so as to establish the optimal concentration to be used. The MTT and colony formation assays showed that 100µM Didox was neither cytotoxic nor cytostatic to these cells during the 24h period of treatment.

Analysis of more than 200 oxidative stress associated genes using mouse oxidative stress, cytotoxicity and NF-κB RT² Profiler™ PCR-Arrays revealed that 100µM Didox treatment of Raw 264.7 macrophages significantly lowered LPS-induced expression of most inflammatory genes (iNOS, IL-1α, IL-1β, IL-6, TNF-α, NF-κB, Cox-2, p38α, TLR4, GM-CSF, G-CSF, CCL2 and CCL4) while
upregulating expression levels of most anti-inflammatory genes (Catalase, glutathione peroxidase, IL-10, superoxide dismutase, and peroxiredoxins) in these cells. Quantitative real-time PCR using selected primers confirmed that Didox inhibited LPS-induced mRNA expression of iNOS, IL-6, IL-1, NF-κB (p65), Cox-2, and TNF-α in Raw cells while upregulating antioxidant related gene (glutathione peroxidase, catalase, and superoxide dismutase) expression after 24h of treatment.

Moreover, using the sandwich ELISA assay, it was demonstrated that Didox dose-dependently inhibits LPS-induced IL-6 production in Raw 264.7 cells. Western blot analysis showed that Didox suppressed LPS-induced protein expression of Cox-2, iNOS, and TNF-α in Raw 264.7 macrophages. The Griess reagent and DAF-2 DA assays showed that Didox inhibits LPS-induced secretion and production of nitric oxide in Raw 264.7 cells. Didox also inhibited LPS-induced reactive oxygen species (ROS) production as assessed with the dihydrodichlorofluorescein diacetate (CM-H_2DCFDA) assay and evaluated using Fluorescence Microscopy Analysis. Didox further blocked Buthionine Sulfoximine (BSO) and LPS-induced ROS production in Raw 264.7 murine macrophages. LPS-mediated oxidative burst and superoxide free radical productions in Raw 264.7 macrophages were also suppressed by addition of Didox as demonstrated using the DHE assay.
In additional functional assays, Didox inhibited both LPS-induced endocytosis of FITC-labeled beads and phagocytosis of TRITC-labeled yeast cells in Raw 264.7 murine macrophages. Immunofluorescence translocation studies with FITC-labeled NF-κB (p65) and Western blot analysis showed that Didox inhibited LPS-induced p65 nuclear translocation in Raw 264.7 cells. These results suggest that the pharmacological action of Didox observed in Raw 264.7 murine macrophages is partly due to its potent anti-oxidative effects and anti-inflammatory potential, and that these actions may be mediated via inhibition of the NF-κB pathway. Didox may therefore play a major in managing disease conditions exacerbated by of LPS-induced oxidative stress in macrophage-mediated inflammation.

**ABBREVIATIONS:** Didox, 3,4-Dihydroxybenzohydroxamic acid; NO, Nitric oxide; iNOS, inducible nitric-oxide synthase; LPS, lipopolysaccharide; NF-κB, nuclear factor-κB, MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; H$_2$DCFDA, 2'7’-dihydrodichlorofluorescein diacetate; BSO, buthionine sulfoximine; IL-6, interleukin 6; ROS, reactive oxygen species; FITC, fluorescein isothiocyanate, MFI, mean fluorescence intensity; DHE, dihydroethidium; DAF-2 DA, 4,5-Diaminofluorescein Diacetate.
INTRODUCTION

Reactive oxygen species (ROS) are a cluster of highly unstable compounds that are generated during inflammation and normal cellular metabolism via incomplete reduction of molecular oxygen. They consist of molecules with unpaired electrons such as superoxide anions ($O_2^-$) and hydroxyl radical ($^\cdot$OH), and molecules that have oxidizing propensity but do not possess free electrons, for example hydrogen peroxide ($H_2O_2$) and hypochlorous acid (HOCl) (Abid et al., 2005). These species are involved in the regulation of fundamental cellular activities such as cell growth and differentiation (Dor and Porat, 2001; D'Angio and Maniscalco, 2002, Hopf et al., 2005, and Veal et al., 2007). However, their overproduction and/or the presence of impaired antioxidant ability results in oxidative stress which can induce and propagate significant injury. Likewise, reactive nitrogen species (RNS), the by-products of nitric oxide (NO) metabolism, play an important role in maintaining various physiological functions at basal levels, and contribute to several pathological processes at high levels. Examples of RNS include nitrite (NO$_2^-$), nitrate (NO$_3^-$) and peroxynitrite (ONOO$^-$) and these are produced in large amounts during the process of inflammation (Boueiz and Hassoun, 2009).

Inflammation is a complex stereotypical response of the body to cell damage and breaches in vascularized tissues. The inflammatory response is phylogenetically and ontogenetically the oldest defense mechanism that is controlled by cytokines, products of the plasma enzyme systems, lipid mediators released from
different cells, and by vasoactive mediators released from mast cells, basophils and platelets (Ross et al., 2002). However, deregulated inflammatory processes induce overproduction of ROS/RNS and reduced cellular antioxidant capacity leading to cellular oxidative stress. Cellular oxidative stress can be a predisposing factor for cancer and inflammation-based disorders since it results in oxidation of DNA, lipid bilayer fatty acids and proteins impairing their function permanently. By oxidizing these vital molecules, oxidative stress can lead to DNA mutation or damage, lipid peroxidation, protein degradation, and these factors have been associated with inflammatory diseases such as atherosclerosis, multiple sclerosis, Parkinson's disease, heart failure, myocardial infarction, Alzheimer's disease, chronic fatigue syndrome, and cancer (Khansari et al., 2009).

A key player in inflammation is the macrophage whose wide tissue distribution makes them well suited to provide an immediate defense against foreign elements prior to leukocyte migration (Zhang et al., 2008). As macrophages participate in both adaptive immunity and innate immunity against bacterial, viral and fungal pathogens, they display a range of functional and morphological phenotypes. The murine macrophage Raw 264.7 cell lines are the best model for these studies since its lipopolysaccharide (LPS) -induced state exhibits these typical functional and morphological phenotypes. LPS triggers the secretion of nitric oxide (NO) and inflammatory cytokines through stimulating intracellular signaling molecules such as Nuclear factor-κB (NF-κB) and mitogen-activated
protein kinases (MAPKs) (Xie et al., 1994). Previous studies have suggested that these LPS-induced pathways play a critical role in LPS-induced iNOS and IL-6 expression (Nam et al., 2007, Cho et al., 2003, Kim et al., 2006).

Nitric oxide, a small-molecule and membrane-permeable gas released by activated macrophages, mediates many physiological events, and chronic elevated nitric oxide is involved in the deamination of DNA and inactivation of DNA repair enzymes, mechanisms that induce cancer (Kim et al., 2007, Kim et al., 2004). Endothelial NO synthase (eNOS), neuronal NO synthase (nNOS), and inducible NO synthase (iNOS) are the three key enzymes that generate nitric oxide. iNOS is responsible for the overproduction of nitric oxide and is often present during inflammation and carcinogenesis (Fukumura et al., 2006). Expression of the iNOS gene in macrophages is regulated mainly at the transcriptional level, particularly by NF-κB (Xie et al., 1994).

In unstimulated cells, NF-κB is constitutively localized in the cytosol as a homodimer or heterodimer, and is associated with inhibitory IκB protein (IκB). Activation of NF-κB is induced by phosphorylation of IκB, leading to its degradation and to the translocation of NF-κB to the nucleus (Griscavage et al., 1996). In this way, NF-κB regulates the inflammatory reaction by increasing the expression of specific cellular genes (Yamamoto et al., 2001). Just like many other NF-κB regulated genes there are NF-κB consensus DNA sequences within the iNOS promoter (Yeo et al., 2003) that are responsible for LPS-induced NF-κB
DNA binding activity. As a result, inhibition of signal transduction proteins in the pathways leading to activation of NF-κB is now widely recognized as an applicable strategy to combat inflammatory disease.

While therapeutic choices for the treatment of commonly occurring chronic inflammatory diseases are coupled with severe side effects, the search for less harmful and effective compounds continues to be a strong research area (McKellar \textit{et al.}, 2007). Didox (3,4-Dihydroxybenzohydroxamine) is a simple, synthetic antioxidant and one of the most potent ribonucleotide reductase inhibitors, revealing an antitumor effect in several experimental studies and has been shown to have the capacity to downregulate NF-κB activation (Elford \textit{et al.}, 2007, Truchan \textit{et al.}, 2003, and Inayat \textit{et al.}, 2002). We have therefore examined the effect of Didox on LPS-induced oxidative stress in Raw 264.7 murine macrophage cell line and show that it possesses potent anti-oxidative and anti-inflammatory effects, and that these actions are mediated via inhibition of the NF-κB pathway. These results suggest that Didox may therefore play a major role in managing disease conditions associated with macrophage-mediated inflammation.
MATERIALS AND METHODS

Materials

Didox was provided by Dr. Howard Elford, Molecules for Health Inc. (Richmond, VA) and phorbol-12-mristate-13-acetate (PMA) was purchased from A.G. Scientific Inc., San Diego, CA. LPS from E. coli serotype R515(Re) (ultra pure, TLR4grade™) was purchased from Alexis biochemicals, San Diego, CA and FBS from Atlas Biological, Fort Collins, CO. Dulbecco’s Modified Eagle Medium (DMEM), fetal bovine serum (FBS), HEPES, glucose, sodium bicarbonate, penicillin, streptomycin, paraformaldehyde, Triton X-100, Sodium Borohydride, buthionine sulfoximine and dimethyl sulfoxide (DMSO Hybri Max®) were purchased from Sigma, St Louis, MO. H₂DCF, DHE, DAF-2 DA (Molecular Probes, Eugene, OR), rabbit anti-65 polyclonal antibody (Thermo Fisher Sci., Fremont, CA), goat-anti rabbit polyclonal Ab, Raw 264.7 murine macrophage cells (ATCC®, Manassas, VA), RT² Profiler™ PCR Array System, RT² First Strand Kit and RT² qPCR Master Mix were purchased from SABiosciences Corp., Frederick, MD, PCR primers from IDT®, Coralville, IA, TRI-reagent® from Molecular Research Center, Inc. Cincinnati, OH and Halt™ Protease Inhibitor Cocktail from Thermo Fisher Sci., Rockford, IL. All other chemicals were purchased from Sigma, St Louis, MO.
Cell Cultures

Murine macrophage RAW 264.7 cells were cultured in Dulbecco’s modified Eagle’s medium (DMEM) containing 25 mM HEPES buffer, 3 g/L D-glucose, 1.5 g/L sodium bicarbonate and 4 mM L-glutamine. The culture media were supplemented with 10% fetal bovine serum, penicillin (100 units/ml) and streptomycin (100 µg/ml). All cultures were maintained in a humidified incubator in 5% CO₂ at 37 °C and cells were be split for sub-culturing two times a week.

Treatment Preparations

Didox (3,4-Dihydroxybenzohydroxamine) was dissolved in dimethyl sulfoxide (DMSO Hybri Max® purchased from Sigma, St Louis, MO) to a final stock concentration of 200 mM and further diluted in culture medium to the working serially diluted concentrations ranging from 3.12 – 200 µM. LPS (E. coli, Serotype R515 ultra-pure, TLR4 grade™ purchased from Alexis Biochemicals, San Diego, CA) was diluted in serum-free culture medium before adding to culture treatments.

Determining cell cytotoxicity using MTT Assay

Cell viability was determined via MTT [3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] assay which is based on the capacity of mitochondrial succinyld dehydrogenase of viable cells to convert the soluble yellow tetrazolium salt into an insoluble formazan product. Briefly 100 µl of cells were seeded at 10⁵ cells/well in 96-well plates and allowed to adhere for 3 h,
thereafter Didox or LPS treatment was added to wells to give the desired final concentrations as indicated under the figure legend. Controls without treatment were run in parallel. Following a 24h or 48h incubation period with or without treatment, 20 µl of a 5 mg/ml solution of MTT (Sigma, St Louis, MO) in phosphate buffered solution (PBS) was added in the medium and the cells were further incubated for 1 h at 37 °C. The culture medium was then completely removed by aspiration and the dark blue crystal formazan resulting from the reduction of MTT was dissolved by adding 200 µl of dimethyl sulfoxide (DMSO) and the plates were left on a shaker for another 20 minutes. The optical density (OD) was measured at 570 nm using a 96-well plate reader (µ-Quant, Bio-Tek Instruments, Inc.) and cell proliferation was estimated as the percentage absorbance of sample relative to control. All treatments and controls were carried-out in triplicates and repeated three times to confirm reproducibility.

Cell staining for apoptotic nuclear morphology using Hoechst/PI double stain.

RAW 264.7 cells were plated on glass cover-slips at a density of $1 \times 10^5$ cells/60-mm dish and allowed to adhere for 3hr. Cells were then treated with media alone or the combination of Didox (100 µM) and LPS (0.1 µg/ml) for a period of 24 hr and then stained with Hoechst/PI regimen. Briefly, at 24 hr after treatment, the cell media were removed by aspiration and the adhered cells were resuspended in 500 µl of Hoechst 33342 dye (5 µg/ml). Cells were incubated for 15 min at 37°C followed by gentle addition of propidium iodide (10 µg/ml). The cells were
then fixed as described above and mounted on glass slides for imaging using epifluorescent microscope.

**Gene Analysis using RT² Profiler™ PCR-Arrays.**

Murine macrophage RAW 264.7 cells were seeded at 5 X 10^6 cells/ml in 75 cm² culture flasks (Corning Inc., NY) and allowed to adhere to flask surfaces to 3h. Cells were then treated with Didox (100 µM) and RNA was extracted from cells after 24h using TRI-reagent® from Molecular Research Center, Inc.( Cincinnati, OH) and retrotranscribed by RT-PCR first strand kit (SuperArray Bioscience Corporation, Frederick, MD). To isolate RNA the cells were scraped from flask surface using BD Falcom™ cell scraper (BD Biosciences, Bedford, MA) and harvested by centrifugation. TRI-reagent® was added (1ml) to all cell pellets and the mixture was homogenized by slow pippeting several times. The homogenate was then incubated at room temperature for 5 min with addition of 0.2 ml chloroform.

After incubating the mixture at room temperature for 15 min, the samples were centrifuged at 12,000 x g, for 15 min at 4 °C. The aqueous phases were transferred to a new 1.5 ml microcentrifuge tube. RNA from the aqueous phase was precipitated by mixing with 0.5 ml isopropyl alcohol with incubation at room temperature for 10 min, and centrifuged at 12,000 x g for 15 min at 4 °C. Precipitated RNA pellets were washed three times with 70% ethyl alcohol, and redissolved in DEPC-treated water. After collecting 1µg RNA from each tube,
Genomic DNA contamination was eliminated by using Genomic DNA elimination mixture (SuperArray Bioscience Corporation, Frederick, MD) and cDNA synthesis was carried using the RT cocktail (SuperArray Bioscience Corporation, MD). Real-time PCR was then performed on BioRad iQ5 real-time PCR detection system (Applied Biosystems) using Mouse Oxidative Stress RT² Profiler PCR Array and RT² Real-Timer SyBR Green/qPCR Master Mix purchased from SuperArray Bioscience Corporation (Frederick, MD). For data analysis the ∆∆Ct method was used; for each gene fold-changes were calculated as difference in gene expression between untreated controls and treated cell cultures. A positive value indicates gene up-regulation and a negative values indicate gene down-regulation.

**RT-PCR validation assays**

To validate the results obtained from the PCR Array, RNA was extracted and cDNA obtained from treated murine macrophages as mentioned above. mRNA levels were analysed by by real-time PCR using the following conditions: 1 cycle at 95°C for 10 min, 40 cycles at 95°C for 15 sec, 55°C for 1 min and quantified using selected primers (Table 2.1). The quantity of these mRNAs was expressed as fold-changes compared to the untreated Raw 264.7 murine macrophage controls using β-actin controls.
Table 2.1: Primer sequences used for Real Time-PCR evaluation of inflammation associated genes in LPS-activated Raw 264.7 cells after treatment with Didox.

<table>
<thead>
<tr>
<th>Gene</th>
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| iNOS  | F: 5’-AATGGCAACATCAGGTCGGCCATCACT-3’  
R: 5’-GCTGTGTGTACAGAAGTCTCGAACTC-3’ |
| COX-2 | F: 5’-GGAGAGACTATCAAGATAGT-3’  
R: 5’-ATGGTCAGTAGACTTTCACA-3’ |
| IL-6  | F: 5’-GAGGATACCAGCCTCAAAGAGAC-3’  
R: 5’-AAAGTCATCATCGTGTTCCATA-3’ |
| p65   | F: 5’-TCATCTCCCCGCAGAGCCAC-3’  
R: 5’-GGGTTTCGGTGATCTCT-3’ |
| GPx   | F: 5’-GGTTCGGCCGCAAATTTA-3’  
R: 5’-CAGGGACCAATGATGTA3’ |
| SOD1  | F: 5’-CGGATGAAGAGAGGCATGGT-3’  
R: 5’-CACCTTTGCGGAAATCT-3’ |
| CAT   | F: 5’-TTGACAGAAGCGGATTCC-3’  
R: 5’-AGGCTGACTCTGACT-3’ |
| TNF-α | F: 5’-GCCAGGGCTGATTAGGA-3’  
R: 5’-TCTCTGCTGCTGACCTT-3’ |
| β-actin| F: 5’-TCATGAAGTCGACCGTCCGT-3’  
R: 5’-CCTAGAACATTTGGCGGACGATG3-3’ |
Measurement of NO production using nitrite assay

For evaluating the possible effect of Didox on LPS-induced NO production, Raw 264.7 cells in MEM (without phenol red) media were plated at a density of $10^5$ cells/well in 96-well culture plate and grown for 3 h to allow them to attach to the plate. Didox was added to the wells at desired final concentrations in the absence or presence of LPS (0.1µg/ml). The cells were further incubated for 24 h. Control cells were grown under identical conditions except without tested compounds. After that, the supernatants were collected for determination of nitrite as an index for nitric oxide (NO) production. NO synthesis in cell culture was measured using Griess reagent. Briefly, 100µl/well of sample was incubated with equal volume of Griess solution (0.1% naphthylethylenediamine and 1% sulfanilamide in 5% H$_3$PO$_4$ solution) at room temperature for 10 min. The absorbance was measured with a 96-well plate reader (µ-Quant, Bio-Tek Instruments, Inc.) at 550 nm. Nitrite concentration was determined by using sodium nitrite as a standard. The level of nitrite, a more stable NO metabolite, reflects NO synthesis since NO has a very short half-life.

Determination of IL-6 production by murine macrophage Raw 264.7 cells

After treatment of cells in the 96-well plates and incubation at appropriate time intervals, cell culture supernatants were removed for cytokine determination assays. The cell culture supernatants were collected and analyzed for IL-6 proteins using commercial ELISA kits Mouse IL-6 ELISA Ready-SET-Go!
(eBioscience, San Diego, CA). The protocols provided by the manufacturers were followed to the detail. Briefly, NUNC Maxisorp 96 well ELISA plates were coated with 100µl/well of capture antibody overnight and after five washes, 100µl/well of detection antibody is added followed by 1h incubation at room temperature. After proper washing, avidin-HRP (horse radish peroxidase) was added at 100µl/well and incubated plate at room temperature for 30 minutes. The avidin-HRP was replaced with substrate solution for 15 minutes and after addition of a stop solution the optical density (OD) can be measured at 450 nm using an ELISA plate reader (µ-Quant, Bio-Tek Instruments, Inc.). IL-6 protein concentration was quantified from a standard curve constructed from serially diluted standard concentration readings in appropriate wells. All treatments and controls were carried in duplicates and repeated three times to confirm reproducibility.

Evaluation of ROS production using fluorescent microscopy

Cells were seeded at 5 x 10^5 cells/well in eight chamber cell culture slides for 3h and then treated with Didox (100µM), BSO (400µM) in the presence or absence of LPS (1µg/ml). After 24h incubation, H_2DCFA solution was added at 10 µM to all wells and incubated for an additional 30 min at 37°C. The slides were washed twice with PBS, fixed with chilled absolute methanol for 1 min and mounted using glycerol:PBS (1:1) solution. The cover slips were placed over the mounting medium and the slides sealed using nail polish. The slides were then viewed
under the fluorescence microscope and images capture using the digital camera with quantification done using the Nikon NIS elements software.

**Assessment of intracellular superoxide radical production in Raw 264.7 cells.**

Superoxide production in Raw cells was assayed by using the oxidative fluorescent dye DHE. DHE is oxidized on reaction with superoxide to ethidium bromide, which mostly binds to DNA in the nucleus and fluoresces red. Cells were seeded on cover slips at 2 x10^5 cells/well (in 6-well plates) for 3hrs. To quench out any residual fluorescence, cells were treated with 0.01% sodium borohydride (in PBS) for 5 min. After loading cells with 10 µM DHE for 15min, cells were treated for 24h with 100 µM Didox in the presence or absence of LPS (0.1µg/ml). Cells were fixed and mounted on slides and analyzed using the Nikon AZ100 Epi-Fluorescent Microscope. The un-oxidized DHE remains blue (λ_{Ex/Em} =355\420nm) while the oxidized DHE fluoresces red (λ_{Ex/Em}=355\420nm). Images were captured using the NIS-Elements Viewer 3.0.

**Intracellular NO production and iNOS activity in Didox-treated Raw 264.7 murine macrophages.**

4,5-Diaminofluorescein Diacetate (DAF-2 DA), can be used for real-time bioimaging of NO with fine temporal and spatial resolution. A membrane-permeable DAF-2 DA is hydrolyzed by cytosolic esterases releasing free DAF-2 DA that does not leak into the medium. At physiological pH, the DAF-2 is relatively non-fluorescent, however, in the presence of NO and oxygen, DAF-2
triazole (DAF-2T), a fluorescent product, is formed. Cells were seeded on cover slips at 2 x 10^5 cells/well (in 6-well plates) for 3hrs. To quench out any residual fluorescence, cells were treated with 0.01% sodium borohydride (in PBS) for 5 min. After loading cells with 10 µM DAF-2 DA for 15min, cells were treated for 24h with 100 µM Didox with or without LPS (0.1 µg/ml). Cells were fixed and mounted on slides and analyzed using the Nikon AZ100 Epi-Fluorescent Microscope.

**Western Blot Analysis**

Murine macrophage RAW 264.7 cells were seeded in 6-well plates to final volume of 5ml at 2.5 x 10^6 cells/ml and incubated at 37ºC for 3 h before treatment with Didox or LPS. After treatment the culture medium was replaced with 500 µl of Lysis Buffer (50 mM Tris base, pH 8, 150 mM NaCl, 1% IGEPAL CA 630, 0.1 % SDS, Protease Inhibitor cocktail) and cells lysed on ice for 30 min with constant shaking. The lysates were then transferred to 1.5 ml microfuge tubes and the insoluble membrane fraction separated from the soluble fraction by centrifuging at 10,000 x g for 10 minutes. The soluble fraction was mixed with sample buffer and boiled for 10 minutes.

Electrophoresis was performed using 4-20% TBE Cititerion™ Precast Gels (Bio-Rad Lab, Inc. Hercules, CA) for 1 hr (200 V, 1 µg protein per lane) in electrode buffer (25 mM Tris base, 190mM Glycine, 0.1% SDS). Separated proteins were transferred to nitrocellulose membranes for 1hr (<100kDa) or overnight
(>

(>100kDa) at 100V or 30V respectively in transfer buffer (25 mM Tris, 190 mM Glycine, pH 8.1-8.4) followed by incubation in 0.01M PBS containing 0.1% Tween 20, 3% BSA, and 0.1% gelatin to block nonspecific IgG binding, followed by treatment with specific primary antibodies for 30-45 min. The membranes are then incubated with alkaline phosphatase-conjugated secondary antibody for 30-45 min. The Western blot analysis was developed using substrate buffer [0.1 M Tris buffer, 0.1M NaCl, 5 mM MgCl₂ pH 9.5, mixed with 1% NBT:BCIP (2:1)].

**Preparation of cytosolic and nuclear extracts**

For cytosolic protein extraction, Raw 264.7 macrophages were resuspended in 500 µL of buffer A (10 mM HEPES, 10 mM KCl, 1 mM MgCl₂, 5% glycerol, 0.5 mM EDTA, 0.1 mM EGTA, 1X protease inhibitor solution, 2 mM PMSF and 0.5 mM DTT) and incubated on ice in slow shaker for 15 min followed by addition of 0.65% of NP-40. The mixture was vigorously vortexed for 10s and centrifuged at 12,000 x g for 1 min. For nuclear protein extraction, the resulting nuclear pellets were resuspended in 500 µL of high salt buffer B (20 mM HEPES, 1% NP-40, 400 mM NaCl, 10 mM KCl, 1 mM MgCl₂, 20% glycerol, 0.5 mM EDTA, 0.1 mM EGTA, 1X protease inhibitor solution, 2 mM PMSF and 0.5 mM DTT) and incubated on ice in slow shaker for 1 hr. The mixture was vigorously vortexed for 15s followed by centrifugation at 12,000 x g for 5 min.
**NF-κB translocation immunofluorescence assay**

Raw 264.7 murine cells were cultured in 8-chamber slides at $2 \times 10^5$ cells/well and seeded for 3hrs before treatment with Didox, LPS or combination of both for 90 min. After fixation with 4% PFA cells were permeablized with 0.1% Triton X-100, 1%BSA for 30 min. Any possible background fluorescence was quenched by incubating cells in 0.01% sodium borohydride (in PBS) for 5 min and non-specific binding sites were blocked by adding 1% BSA with 1 hr incubation. Cells were incubated for 60 min with rabbit anti-p65 antibody (1:1000) (purchased from Thermo Fisher Sci., Fremont, CA) followed by FITC-labeled goat anti-rabbit secondary Ab incubation for 60 min. After 5 min nuclear staining with DAPI cells were mounted on slides using 50% glycerol and analyzed using the Nikon AZ100 Epi-Fluorescent Microscope. Images were captured using the NIS-Elements Viewer 3.0.

**WEHI Bioassay for TNF-α production**

The production of TNF-α by Raw 264.7 macrophages can be evaluated further using a bioassay which measures the cytotoxicity of this cytokine on TNF-α sensitive WEHI-164 cells. The WEHI 164 fibrosarcoma cell line assay described below can be used for assaying TNF-α by measuring cell survival after treatment with supernatants obtained from Raw 264.7 cells. TNF-α induces cell death via apoptosis in WEHI-164 cells and this assay was used to analyze the production and secretion of bioactive TNF-α. Raw 264.7 murine macrophages were treated with Didox, LPS or their combination for 24h and the supernatants were
collected. WEHI-164 cells were cultured cells in 96 well plates at 5 x 10^4 cells/well and incubated for 2-3h in 37°C humidified incubator to allow adherence to the plate surface. Several 1:5 dilutions of supernatants collected from Raw cells conditioned media were added in triplicate wells containing seeded WEHI cells and further incubated for 24h in 37°C humidified incubator with 5% CO₂. Afterwards the media was aspirated and replaced with MTT working solution (5 mg/ml) and incubated for incubate for 4h at 37°C until the blue formazan was visible. The MTT was then aspirated and DMSO was added to solubilize the formazan on shaker for 10 min. The absorbance was read at 570nm and the percentage of surviving cells was calculated in comparison with control cells. Control cells were established by treating WEHI cells with supernatants obtained from untreated Raw 264.7 cells. The assay was repeated three times and a representative image of cells was photographed using the Nikon AZ100 Microscope.

**Bioassay for IL-6 production using B9 cell lines**

The production of IL-6 by Raw 264.7 macrophages was evaluated using a bioassay which measures the proliferative effect of this cytokine on IL-6 dependent B9 cell lines. The IL-6 dependent murine hybridoma cell line B9 provides a reliable and sensitive assay for measuring IL-6 production in mammalian cultures. B9 cells were cultured in RPMI-1640 medium supplemented with 10% FBS and IL-6. For the bioassay, B9 cells were washed and seeded to a density of 2.5 x 10^4 cells/well in 96-well microtiter plates. A 1:5
dilution of supernatants obtained from Didox (100 µM) and LPS (0.1 µg/ml) treated Raw cells was added in triplicates and incubated for 24h in a 37°C humidified incubator with 5% CO₂. B9 cells treated with cultured media alone were used as negative controls. Afterwards the plates were centrifuged at 500 x g for 10 min to pellet cells and media was aspirated to be replaced with MTT working solution (5 mg/ml) and incubated for 4h at 37°C until the blue formazan was visible. The MTT was then aspirated and DMSO was added to solubilize the formazan on shaker for 10 min. The absorbance was read at 570nm and the stimulation index was calculated in comparison with control cells. Control cells were established by treating B9 cells with supernatants obtained from untreated Raw 264.7 cells. The assay was repeated three times and a representative image of cells was photographed using the Nikon AZ100 Microscope.

**Phagocytosis Assay (Microscopic analysis using FITC-beads)**

For determination of effect of Didox of macrophage functionality, Raw 264.7 murine macrophages were seeded at 10⁵ cells/well in 8-chamber glass slides and incubate for 3hrs followed by treatment with LPS (0.1µg/ml), CytA (1 µM) and Didox (100µM) for 30 min. FITC-labelled beads were added at 0.1 mg/ml or 10⁶ particles per well and incubated for 30 min in 37°C incubator. After washing with PBS, 100 µl Trypan blue (2 mg/ml in 20 mM citrate, 150 mM NaCl, pH 4.5) was added to quench extracellular FITC beads for 2 minutes. Cells were fixed by incubating in 4% PFA for 30 min and mounted onto coverslip using 50 % glycerol
Sealing was done using colorless clear nail polish and cells were evaluated using the Nikon AZ100 Epi-Fluorescent Microscope and images captured using the NIS-Elements Viewer 3.0. The percentage of phagocytosis of FITC-labeled beads was quantified microscopically by counting at least 100 cells in randomly selected fields per well, and an average between the duplicate wells was calculated. Phagocytic index was calculated as: (average number of beads phagocytosed per macrophage) x (% macrophages that had phagocytosed one or more beads).

**Statistical Analysis**

All experiments are representative of three repeats carried out in triplicates and the error bars represent the standard deviation from the mean average. All data representations were analyzed using Graphpad PRISM 4.0 (Graphpad, San Diego, CA, USA) or Sigmaplot (SPSS). p<0.05 would be considered to be statistically significant.

**RESULTS**

**Determination of effect of Didox on Raw 264.7 cell viability.**

The effect of Didox on Raw 264.7 cell viability was determined by the mitochondrial-dependent reduction of yellow MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazoliumbromide] to blue formazan. Raw 264.7 murine macrophages were seeded at $10^5$ cells/well in 96-well plates for 3h and then
treated with increasing concentrations of Didox (0-200µM) in the presence or absence of LPS for 24h. Replacing the media with MTT solution revealed that cell viability was maintained above 90% at the concentrations tested with no significant indication of cytotoxicity (Fig 2.1) and the PI exclusion assay showed that cells exhibited no signs of apoptosis (Fig 2.2). To further evaluate cell integrity and long-term effect of Didox on Raw 264.7 murine macrophages, microscopic images of cells were captured at 12h intervals for up to 72h with no significant change in cell turnover or morphology (Fig 2.3).
Figure 2.1. Effect of Didox on cell viability of LPS-stimulated Raw 264.7 murine macrophages. Raw 264.7 murine macrophages were incubated with Didox (0-200µM) and LPS (0.1µg/ml) for 24h and cell viability was quantified using the MTT assay. Each value represents the mean ± SD of three experiments performed in triplicates.
Figure 2.2. Didox-treated Raw 264.7 macrophages exclude PI dye and exhibit no signs of apoptosis during 24h period. Raw 264.7 murine macrophages were seeded on coverslips and incubated with Didox (100µM) and LPS (0.1µg/ml) for 24h followed by incubation with PI/Hoechst 33342 stain. Each value represents the mean ± SD of three experiments performed in triplicates. After mounting, slides were viewed under fluorescent microscope and images captured using Nikon NIS Elements viewer.
Figure 2.3. Didox exhibits no cytostatic effect on Raw 264.6 cells for up to 48h of treatment. Raw 264.7 macrophages were seeded at $5 \times 10^4$ cells/well on coverslips in 6-well plates and incubated with Didox (100µM) and LPS (0.1µg/ml) for up to 48hr and growth was monitored every 12h using microscope DIC filter and images captured using Nikon NIS Elements viewer.
Analysis of oxidative stress associated genes using mouse oxidative stress, cytotoxicity and NF-κB RT² Profiler™ PCR-Arrays.

Quantification of oxidative stress-associated genes is an important element in studies of inflammation and immune responses. Quantitative RT-PCR, a rapid and sensitive assay, is the preferred method to quantify gene mRNA levels because they are often expressed at low levels. The PCR Array System offers a simple, reliable and sensitive tool for multiple gene profiling. Using the Mouse Oxidative stress and NF-κB PCR Array, the mRNA levels of 84 different oxidative stress-related genes in LPS-stimulated and untreated Raw 246.7 murine macrophage cells versus their Didox-treated (100µM) counterparts were monitored. The gene expression results identified a number of up-regulated and down regulated genes upon 24 h of treatment. At 24 hours, the positive effects of LPS on pro-oxidant genes such as CSF’s, Chemokines, TLR’s, IL1α, IL1β, IL6, iNOS, NF-κB(p105) and TNF were observed, while its inhibitory effect on anti-oxidant related genes such as IL-10 and Gpx2 was evident twenty-four hours after stimulation (Table 2.2). Didox treatment lowered expression levels of most inflammatory genes while upregulating expression levels of most anti-inflammatory genes.
Table 2.2 A-D. Downregulation of oxidative stress associated mRNA gene levels by Didox in LPS-stimulated raw 264.7 murine macrophages. The Mouse Oxidative stress and NF-κB PCR Array was used to evaluate the mRNA levels of 84 different oxidative stress-related genes in Raw 246.7 murine macrophage cells treated with LPS (0.1µg/ml), Didox (100µM), or both. PCR was performed on BioRad iQ5 real-time PCR detection system as indicated in the methods. For data analysis the ∆∆Ct method provided by SABioscience was used; for each gene fold-changes were calculated as difference in gene expression between untreated controls and treated cell cultures. A positive value indicates gene up-regulation and a negative value indicates gene down-regulation.

### A. Oxidative stress associated genes

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### B. NF-κB transcription factor associated genes

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Validation of RT-PCR array results of selected genes using specific primers.

To validate the results obtained using the RT² Profiler™ PCR-Arrays, an additional RT-PCR analysis was carried out using selected primers to evaluate the expression levels of genes involved in inflammatory pathways. These RT-PCR assays showed that LPS-treatment increases mRNA expression levels of NF-κB (p65), TNF-α, iNOS, IL-6, and Cox-2 genes (Fig 2.4A-E). On the other hand, addition of Didox reduced expression of these LPS-induced mRNA expression levels after 24h of treatment. Didox treatment of Raw 264.7 murine macrophages led to upregulated mRNA expression levels of antioxidant-related genes such as Gpx, Cat, SOD (Fig 2.4F-H).
Figure 2.4: Real-time PCR data showing modulation of antioxidant and oxidative stress associated mRNA gene levels in LPS-stimulated Raw 264.7 murine macrophages by Didox. RT-PCR was conducted using cDNA from LPS-stimulated Raw 264.7 murine macrophages treated with Didox (100 µM) for 24h. Primers used are outlined in Table 1. LPS-treatment increases mRNA expression levels of iNOS (A), IL-6 (B), TNF-α (C), Cox-2 (D), and NF-kB(E) with variable inhibition by Didox. Didox treatment upregulated mRNA expression of Gpx1 (F), SOD1 (G), and Cat (H). Each value represents the mean ± SD of three experiments performed in triplicates using β-actin as the PCR control.
Didox inhibits LPS-induced NO secretion in Raw 264.7 murine macrophages.

Activated macrophages produce NO in large amounts during LPS-stimulation and inflammatory responses. Since NO production is mediated by iNOS and RT-PCR results indicated an inhibitory role of Didox on iNOS mRNA expression, it was important to evaluate the effect of Didox on NO production in these LPS-activated macrophages. To assess the effect of Didox on LPS-induced NO production in RAW 264.7 macrophages, cell culture medium was harvested after treatment, and the concentration of accumulated nitrite, the oxidative product of NO, was determined by the Griess method. Didox significantly (p<0.01) inhibited LPS-induced NO release in RAW 264.7 cells in a concentration-dependent manner (Fig 2.5). Didox at 100µM significantly decreased NO production while maintaining cell viability of Raw 264.7 macrophages around 100% and although 200µM Didox decreased NO production further, cell viability was also slightly lowered.
Didox suppresses intracellular NO production and iNOS activity in LPS-induced Raw 264.7 murine macrophages.

For direct measurement of intracellular production of NO in cells, 4,5-Diaminofluorescein Diacetate (DAF-2 DA), can be used for real-time bioimaging.
of NO with fine temporal and spatial resolution. Cells were treated with Didox or LPS and then loaded with the membrane-permeable DAF-2 DA which is subsequently hydrolyzed by cytosolic esterases releasing free DAF-2 DA that does not leak into the medium. At physiological pH, the DAF-2 is relatively non-fluorescent, however, in the presence of NO and oxygen, DAF-2 triazole (DAF-2T), a fluorescent product, is formed. The fluorescent images showed that treatment of cells with LPS produced more DAF-2 fluorescence compared to control and Didox addition inhibited LPS-induced fluorescence or NO production in Raw 264.7 cells (Fig 2.6 & Fig 2.6b).

Dose-dependent reduction of LPS-induced IL-6 production in Raw 264.7 murine macrophages by Didox.

Interleukin-6 is one of the major inflammatory cytokines produced and secreted by LPS-activated macrophages. A sandwich ELISA assay was employed to measure the effect of Didox on IL-6 secretion in Raw 264.7 murine macrophage cells since Didox inhibitory effect was observed on IL-6 mRNA expression using RT-PCR. Cells were incubated with LPS (0.1 µg/ml) or Didox (0, 6.25, 12.5, 25, 50, 100, and 200 µM) and culture medium harvested after 24h and analysed for IL-6 production using Mouse IL-6 ELISA Ready-SET-Go! Kit. Didox significantly decreased IL-6 production in a dose-dependent fashion (p<0.01) as compared to the LPS-stimulated cells. Didox at 100 µM reduced IL-6 production ten-fold after 24 h (Fig 2.7). The B9 bioassay for IL-6 production also confirmed that Didox inhibits IL-6 production in LPS-stimulated Raw cells. When supernatants from the
Figure 2.6. Assessment of intracellular NO production using DAF-2 DA detection system in Raw 264.6 cells treated with LPS in the presence or absence of Didox. Cells were seeded on cover slips at $2 \times 10^5$ cell/well (in 6-well plates) for 3hrs. After loading cells with 10 µM DAF-2 DA for 15min, cells were treated with Didox (100µM) or LPS (1µg/ml). DAF-2T product fluoresces green upon reaction with NO. Cells were fixed and mounted on slides and analyzed using the Nikon AZ100 Epi-Fluorescent Microscope.
Figure 2.6b. Quantitative assessment of intracellular NO production using DAF-2 DA detection system in Raw 264.6 cells treated with LPS in the presence or absence of Didox. Cells were seeded on cover slips at 2 x 10^5 cell/well (in 6-well plates) for 3hrs. After loading cells with 10 µM DAF-2 DA for 15min, cells were treated with Didox (100µM) or LPS (1µg/ml). DAF-2T product fluoresces green upon reaction with NO. Cells were imaged using Nikon AZ100 Epi-Fluorescent Microscope under 488nm fluorescence wavelength, DIC-high, and the two were overlayed using the NIS-Elements Viewer 3.0. Mean fluorescence intensities were measured in various fields in triplicates and treatment effects were analysed using the paired t-test and p<0.05 (*) was considered to be statistically significant compared to controls (unless otherwise indicated)
Figure 2.7. Didox inhibits IL-6 production in LPS-stimulated Raw 264.7 murine macrophages. Raw 264.7 murine macrophages were incubated with Didox (0-200µM) and LPS (0.1µg/ml) for 24h and IL-6 production was measured from collected supernatants using the ELISA Ready-SET-Go Kit. Each value represents the mean ± SD of three experiments performed in triplicates. (*) indicates p<0.05 vs. the LPS-treated control group not treated with Didox.
Figure 2.7b. Bioassay for IL-6 using the B9 cell line confirms that Didox inhibits IL-6 production in LPS-stimulated Raw 264.7 murine macrophages. Raw 264.7 murine macrophages were incubated with Didox (100µM) and LPS (0.1µg/ml) for 24h and collected supernatants diluted 1:5 were used to treat WEHI cells for 24h. Cell viability was detected using the MTT assay and the stimulation index was determined by dividing OD_{570nm} (sample) with OD_{570nm} (Control). After pelleting cells in the 96-well plate a representative image of cells was photographed using the Nikon AZ100 Microscope.
Didox and LPS treated Raw cells were used for treatment of WEHI cells, supernatants from DX treated cells resulted in minimal B9 growth compared to LPS counterparts. Supernatants from Raw cells treated with combination of DX and LPS resulted in lower B9 growth stimulation than those from LPS-treated cells (Fig 2.7b).

**Didox inhibits H$_2$O$_2$ production in Raw 264.7 murine macrophages treated with BSO, PMA, LPS or their combinations.**

During inflammatory reactions, activated macrophages produce H$_2$O$_2$ in large amounts. Catalase is a major antioxidant enzyme catalyzing the decomposition of hydrogen peroxide (H$_2$O$_2$) and its mRNA levels were upregulated by Didox as shown using RT-PCR. H$_2$O$_2$ production in Raw 264.7 cells was therefore determined using H$_2$DCF (2′,7′-dichlorodihydrofluorescein diacetate) assay after treatment with Didox (100µM), buthionine sulfoxamine, BSO (400µM) in the presence or absence of LPS (0.1µg/ml). H$_2$DCF is a cell-permeant fluorogenic indicator for reactive oxygen species (mainly H$_2$O$_2$) that is non-fluorescent until the acetate groups are removed by intracellular esterases and oxidation occurs within the cell in the presence of H$_2$O$_2$. Higher intracellular fluorescence was observed in BSO and LPS or PMA-treated cells compared to untreated controls. Didox (100 µM) inhibited H$_2$O$_2$ production in both BSO and LPS or PMA-treated cells as indicated by low intracellular DCFA fluorescence compared to BSO and LPS-treated cells (Fig 2.8A and 2.8B). Mean fluorescence intensities were
quantified using the Nikon NIS-Elements Viewer software and confirmed higher mean fluorescence intensities in BSO and LPS or PMA-treated cells compared to Didox-treated cells (Fig 2.8C).
Figure 2.8. Didox inhibits ROS generation in BSO and LPS-stimulated Raw 264.7 murine macrophages. Cells were seeded at $5 \times 10^5$ cells/well in eight chamber cell culture slides for 3h and then treated with Didox (100µM), in the presence or absence of LPS (1µg/ml) [A], BSO (400µM) [B] or combination. After 24h incubation, H$_2$DCF solution was added to all wells and incubated for an additional 30 min at 37°C. Cells were imaged using Nikon AZ100 Epi-Fluorescent Microscope under 488nm fluorescence wavelength, DIC-high, and the two were overlayed using the NIS-Elements Viewer 3.0. Mean fluorescence intensities were measured [C] in various fields in triplicates and treatment effects were analysed using the paired t-test and $p<0.05$ (*) was considered to be statistically significant compared to controls (unless otherwise indicated).
Didox inhibits production of superoxide radicals by LPS- and PMA-activated RAW 264.7 macrophages

Since RT-PCR analysis showed an up-regulation of SOD in Raw 264.7 cells treated with Didox it was important to assess the intracellular levels of superoxides in these cells after treatment with Didox. To achieve this, cells were treated with Didox in presence or absence of LPS for 24h and then loaded with DHE for 2h in presence or absence of PMA. DHE-positive cells fluoresce red while unoxidized DHE remains blue. DHE-positive cells were assessed using a fluorescent microscope and superoxide production was quantified by measuring fluorescence intensity. Treatment of Raw 264.7 cells with LPS and PMA resulted in robust intracellular $\text{O}_2^\cdot$ production whereas addition of Didox significantly decreased $\text{O}_2^\cdot$ production (Figure 2.9, 2.10 and 2.10b).
Figure 2.9. Didox inhibits production of superoxide radicals by LPS-activated RAW 264.7 macrophages. Cells were treated with Didox in presence or absence of LPS for 24h on glass coverslips and then loaded with DHE for 2h. DHE-positive cells fluoresce red and localize mostly in the nucleus while unoxidized DHE remains blue.
Figure 2.10. Didox inhibits production of superoxide radicals by PMA-activated RAW 264.7 macrophages. Cells were treated with Didox or PMA in presence or absence of BSO for 24h on glass coverslips and then loaded with DHE for 2h. DHE-positive cells fluoresce red and localize mostly in the nucleus while unoxidized DHE remains blue.
Figure 2.10b. Quantitative analysis of production of superoxide radicals by LPS-activated RAW 264.7 macrophages after treatment with Didox. Cells were treated with Didox, LPS or PMA in presence or absence of BSO for 24h on glass coverslips and then loaded with DHE for 2h. DHE-positive cells fluoresce red and localize mostly in the nucleus while unoxidized DHE remains blue. Mean fluorescence intensities were measured in various fields in triplicates and treatment effects were analysed using the paired $t$-test and $p<0.05$ (*) was considered to be statistically significant.
Inhibition of LPS-mediated NF-κB(p65) nuclear translocation in Raw 264.7 murine macrophages by Didox.

The production of most inflammatory proteins is regulated through the NF-κB pathway and the RT-PCR mRNA expression assay showed that Didox has an effect on NF-κB expression. To define the molecular action by which Didox affects NF-κB activation pathway in Raw 264.7 murine macrophages, the cells were incubated with Didox (100 μM), LPS (0.1 μg/ml) or both for 24h and NF-κB (p65) levels. NF-κB translocation was evaluated using immunofluorescence microscopy by observing presence of p65 in cytosol or nuclei. More fluorescence was observed in the cytosol than in nuclei of untreated and Didox treated Raw 264.7 murine macrophages whereas the reverse was observed in LPS-treated cells (Fig 2.11). Didox also reduced p65 presence in nuclei since less fluorescence was observed in nuclei of LPS-stimulated cells treated with 100 μM Didox while more fluorescence occurred in the cytosol. The presence of more p65 in cytosol of LPS-treated cells indicated that Didox inhibited translocation of LPS-induced NF-κB nuclear translocation.

These results were confirmed using Western blot analysis which showed high presence of p65 protein levels in LPS-treated cells than in Didox-treated cells (Fig 2.12). The presence of high p65 protein levels in nuclei of cells stimulated with LPS was diminished by co-treatment with 100 μM Didox indicating the inhibitory action if Didox on LPS-mediated NF-κB nuclear translocation in Raw cells.
Figure 2.11. Didox inhibits LPS-mediated NF-κB (p65) nuclear translocation in Raw 264.7 cells. Cells were incubated with LPS (0.1µg/ml) and/or Didox (100µM) for 24h and NF-κB translocation was evaluated using immunofluorescence microscopy by observing presence of p65 in cytosol or nuclei using FITC-labeled Ab. Cells were imaged using Nikon AZ100 Epi-Fluorescent Microscope under FITC wavelength and mean fluorescence intensities were measured in various fields in triplicates and treatment effects were analysed using the paired t-test and p<0.05 (*) was considered to be statistically significant compared to controls (unless otherwise indicated). See complete image below in Fig 2.11b with all fluorescence channels.
Figure 2.11b. Complete view of NF-κB nuclear translocation immunofluorescence in LPS-activated Raw 264.7 cells treated with Didox. The figure shows DAPI, DIC, FITC channels and their merged images.
Figure 2.12. Didox inhibits LPS-mediated NF-κB (p65) nuclear translocation in Raw 264.7 cells. Cells were incubated with LPS (0.1µg/ml) and/or Didox (100µM) for 24h and NF-κB translocation was evaluated using western blot analysis after extraction of cytosolic and nuclear proteins.
Didox inhibits LPS-mediated protein expression of iNOS, Cox-2 and TNF-α in Raw 264.7 cells.

Among the target inflammatory genes regulated by NF-κB activation, iNOS, Cox-2, and TNF-α are central to onset and progression of macrophage-mediated inflammation. It was therefore essential to determine if Didox’s ability to inhibit NF-κB activation can influence protein expression levels of iNOS, Cox-2 and TNF-α. To achieve this goal Raw 264.7 macrophage protein expression levels of iNOS, Cox-2 and TNF-α were evaluated using the Western blot analysis after 24 h of treatment with Didox (100 µM) in the presence or absence of LPS. Didox suppressed LPS-mediated protein expression of iNOS, Cox-2 and TNF-α in Raw 264.7 macrophages after 24h (Fig 2.13). Supernatants from LPS-treated Raw cells were very toxic to WEHI cells during the WEHI bioassay for TNF-α production. Didox lowered this toxicity resulting in more WEHI survival since supernatants from Didox-treated Raw cells showed a higher WEHI cell survival compared to those from LPS-treated cells (Fig 2.13b).

Didox suppresses phagocytosis on FITC-labeled latex beads by LPS and PMA activated Raw 264.7 murine macrophages

To evaluate the effect of Didox on macrophage functionality, cells were incubated with FITC-labeled beads after treatment with Didox in the presence or absence of LPS or PMA as outlined above. Efficiency of phagocytosis was determined by using the phagocytosis index calculated as described above. Engulfment of
beads increased with LPS priming while cells treated with Didox engulfed fewer beads than untreated controls. Didox also reduced engulfment of FITC-labeled latex beads in LPS- and PMA-treated Raw 264.7 as fewer latex beads were internalized in Didox-treated cells (Fig 2.14).

Figure 2.13. Didox inhibits LPS-mediated protein expression of iNOS, Cox-2 and TNF-α in Raw 264.7 cells. Cells were incubated with LPS (0.1µg/ml) and/or Didox (100µM) for 24h and iNOS, Cox-2, and TNF-α protein expressions were evaluated using western blot analysis using β-actin house keeping gene to equalize loading.
Figure 2.13b. WEHI bioassay for TNF-α production confirms that Didox inhibits LPS-mediated production of bioactive TNF-α in Raw 264.7 cells. Raw cells were incubated with LPS (0.1µg/ml) and/or Didox (100µM) for 24h and supernatants were collected for use (1:5 dilution) in treating WEHI cells for 24h. Cell survival was determined by comparison with control cells.
Figure 2.14. Didox inhibits uptake of FITC-latex beads in LPS-stimulated Raw 264.7 cells. Cells were treated with Didox (100µM) or LPS (0.1µg/ml) and combination of both for 1h. FITC-labeled beads were added to each well and bead-uptake was evaluated using immunofluorescence microscopy and images were analyzed using Nikon AZ100 Epi-Fluorescent Microscope under FITC wavelength, DAPI wavelength, DIC-High and the three were overlayed using the NIS-Elements Viewer 3.0 (Fig 2.14b below shows the complete image with the 3 channels and overlays).
Figure 2.14b. Complete view of FITC-labeled beads in Raw 264.7 cells using DAPI, DIC, FITC channels, and their merged images.
The effect of Didox on filamentous actin (F-actin) polymerization and uptake of TRITC-labeled yeasts by Raw 264.7 murine macrophages.

Uptake and killing of microorganisms by professional phagocytes are dependent on local, rapid remodeling of actin and immediately after ingestion phagosomes are surrounded by a distinct rim of filamentous actin (F-actin). To evaluate the effect of Didox on this F-actin reorganization phenomenon and its role in phagocytosis, cells were challenged with TRITC-labeled yeasts (S. cerevisiae) with concomitant staining of F-actin using FITC-labeled phalloidin. Cells were then fixed and analyzed using epi-fluorescent and confocal microscope. Although untreated Raw macrophages engulfed more yeast cells, addition of Didox to untreated and LPS-treated cells resulted with a significant decrease in yeast-uptake (Fig 2.15). Staining of F-actin revealed that the actin cytoskeletal reorganization was robust and extensive without Didox treatment and a rim of F-actin located around the engulfed yeast particles. Although more extensions and striated villi were observed in the absence of Didox than when Didox was added, the actin cytoskeletal structure formation and organization was only slightly affected by Didox.
Figure 2.15. Didox inhibits uptake of TRITC-labeled yeasts by Raw 264.7 macrophages. Cells were seeded on coverslips in 6-well plates and incubated with TRITC-labeled yeasts in presence or absence of Didox for 30 min.
DISCUSSION

The use of non-steroidal anti-inflammatory drugs that possess intrinsic anti-inflammatory and antioxidant properties while triggering the intracellular cascades of protective pathways offers a promising strategy for therapeutic applications in macrophage-mediated aberrant inflammation. An interesting agent that might fulfill these criteria and inhibit excessive oxidative stress in macrophages is the polyhydroxyphenyl hydroxamic acid derivative, Didox (N-3,4-trihydroxybenzamide), which has been shown to possess antioxidant, oxygen scavenging, and anti-inflammatory properties in a variety of experimental systems. As a result, studies to explore the anti-inflammatory effects of Didox on LPS-induced oxidative stress and inflammation in Raw 264.7 murine macrophages were a central focus area of this study. Didox comprise a new class of antioxidants with ability to inhibit ribonucleotide reductase activity, act as chelators of iron, and scavenge free radicals (Ding et al., 1992; Elford and Riet, 1989).

Didox was also previously shown to inhibit a preeminent inflammatory transcription factor, NF-κB, probably due to their selective inhibition of phosphorylation of the cytoplasmic inhibitor IκBα (Lee et al., 1997). Whether this interesting molecule has other molecular effects that are potentially therapeutic is unknown. It was therefore important to study the anti-inflammatory effect of Didox in the LPS-stimulated Raw 264.7 murine macrophages and propose the
molecular mechanism that could be involved in governing the resulting immuno-modulatory effects.

The evaluation of Didox’s potential anti-inflammatory effects was carried-out using the LPS-inducible Raw 246.7 murine macrophage model system. The Raw 264.7 murine macrophage cell line is most suitable for these studies because of its morphological and functional similarity to human macrophage system. This cell line is sensitive to LPS derived from gram-negative bacteria and thus provides an inducible form of macrophages that undergo phagocytosis and secrete lysozyme upon activation. This allows the evaluation of Didox on gene profiles, morphological outlines, functional characteristics and inflammatory behavior of macrophages in the activated and non-activated forms.

The first undertaking was to evaluate the cytotoxic effect of Didox on Raw 264.7 macrophages and find an optimal concentration of Didox suitable for modulating the inflammatory behavior of Raw 264.7 murine macrophages. This was done by subjecting Raw cells to increasing concentrations of Didox for 24h and determining the cells’ viability profiles. The MTT assay revealed that Didox doses up to 100µM do not cause any significant reduction in cell death of Raw 264.7 macrophages and this was further confirmed by using the PI dye exclusion assay and Flow cytometry assay using PI and Annexin-V. Together these set of results show that both LPS (0.1µg/ml) and Didox (100µM) do not significantly
inhibit cell proliferation or induce any form of cell death in Raw 264.7 murine macrophages during 24h of treatment. Didox is known for its inhibitory role on ribonucleotide reductase, an enzyme that catalyses the reduction of all four ribonucleotides to deoxyribonucleotides for use in DNA synthesis. This RR inhibitory property of Didox warrants an investigation into the potential cytostatic action of this drug on Raw 264.7 cells. Differential interference contrast (DIC) microscopic images were taken at 12h intervals for up to 48h to evaluate cell growth profiles and colony formation after treating cells with LPS, Didox or in combination. The cell growth profiles were not significantly affected by Didox or LPS during the 48h period, suggesting that Didox does not exert a significantly noticeable cytostatic action on these Raw cells.

There is a wide variety of genes and pathways that play a pivotal role in regulation or generation of oxidative stress in activated macrophages; hence it was therefore necessary to start by identifying the effect of Didox on expression of genes associated with inflammation and oxidative stress in LPS-activated Raw 264.7 macrophages. This was achieved through a multi-gene profiler array system that assayed a total of more than 200 genes involved in Raw 264.7 inflammatory behavior. These PCR arrays are the most reliable tools for analyzing the expression of a focused panel of genes with high reproducibility, sensitivity and specificity. Raw 264.7 cells were treated with LPS, Didox, or combination of both for 24h and mRNA isolated for gene expression studies and
several oxidative stress and inflammation-related genes significantly affected by Didox were indentified. Multigene profiling with real time PCR Array Profiler revealed Didox-mediated inhibition of several inflammatory genes involved in LPS-induced macrophage inflammatory activities and these included chemokines (CCL2, CCL3, CCL4, CXCL10), IL-1, IL-6, TNF-α, iNOS, NADPH oxidase, GM-CSF (Csf2), G-CSF (Csf3), Akt1, NF-κB (p105), and TLR4. Didox treatment also resulted in increased mRNA gene expression of catalase, glutathione peroxidase, and superoxide dismutase as determined by multigene superray profiler. Once the effect of Didox on these genes was noticed, it was therefore required to carry-out a PCR validation assay using specific primers targeting the selected genes. The PCR validation assay confirmed that Didox suppressed mRNA expression of IL-6, TNF-α, iNOS, Cox-2, p38α, p65, and p21.

Taking a closer look at these Didox-suppressed gene: TNF-α is known to be a key mediator for the induction of apoptosis and development of humoral immune response. However, at high concentrations, TNF-α has more pronounced disadvantageous effects, such as inducing tissue injury, oxidative stress, inflammation and potentiating septic shock (Shohami et al., 1999). Elevated serum IL-6 levels have been observed in a number of pathological conditions, including bacterial and viral infections, trauma, autoimmune diseases, and inflammations (Hirano et al., 1994). iNOS catalyses the formation of NO, a highly reactive free radical involved in a number of physiological and pathological
processes. The role of NO is well established in the relaxation of vascular smooth muscles, in the inhibition of mitogenesis and growth of glomerular mesangial cells, and in macrophage toxicity. Under normal physiological conditions NO also acts immunologically as a cytotoxic agent on invading microorganisms in macrophages or on tumor cells. Oxidative stress and inflammatory responses in activated Raw 264.7 cell line have been linked to increase in mRNA expressions of inflammation-related genes such as IL-1, IL-6, TNF-α, iNOS, and COX-2 induced via the NF-κB pathway (Park and Park, 2009). Thus, it is well documented that these above-mentioned Didox-suppressed genes play a pivotal role in induction of cellular oxidative stress and inflammation in macrophages and their inhibition opens possibilities for Didox as a regulator of oxidative stress and inflammation.

IL-6 has often been linked to inflammatory as well as autoimmune diseases and influences a plethora of cellular functions. IL-6 has also been well demonstrated to be among the strong mediators of oxidative stress and has the potential to alter redox equilibrium (Nussler et al., 1992, Chen et al., 1998, Desmarquest et al., 1998). This prompted us to investigate the effect of Didox on production of IL-6 in LPS-activated Raw 264.7 murine macrophages. Treatment of cells with Didox led to a dose-dependent reduction of IL-6 secretion by LPS-stimulated macrophages with maximal inhibition attained at 100 µM concentration of Didox. These results correlated with the real-time PCR evaluation assays which showed that Didox reduced LPS-mediated mRNA expression of IL-6. Didox could,
therefore, be exerting its inhibitory effects on ROS generation via inhibition of IL-6 production thereby reducing the LPS-induced inflammatory activity of Raw 264.7 murine macrophage cell lines.

Results from RT-PCR validation assays also confirmed that Didox-mediated inhibition of inflammatory genes in LPS-activated Raw 264.7 macrophages was accompanied by up-regulation of antioxidant enzymes: catalase (Cat), glutathione peroxidase (Gpx), and superoxide dismutase (SOD). Since oxidative stress is a result of a perturbed balance between ROS production and antioxidant levels, the up-regulation of antioxidant pools with concomitant inhibition of oxidative stress genes is very important in shifting the oxidative stress status to the homeostatic stage.

Inhibition of iNOS mRNA expression warranted an investigation on iNOS activity by determining if Didox inhibits NO secretion in LPS-stimulated Raw 264.7 murine macrophage cell line. Using the Griess assay it was shown that Didox dose-dependently inhibits LPS-induced NO secretion. To further determine intracellular NO production, a vital dye sensitive for NO was used to track NO levels after treatment of LPS-activated Raw cells with Didox. DAF-2DA fluorescence was robust in LPS-treated cells compared to untreated controls and this NO sensitive fluorescence was ablated with addition of Didox, confirming the ability of Didox to suppress intracellular NO production.
Other ROS and RNS mediators that induced oxidative stress include \( \text{H}_2\text{O}_2 \), superoxide radicals, and nitrogen peroxides. Evaluation of intracellular ROS production using a fluorescein marker indicated that \( \text{H}_2\text{O}_2 \) increased with addition of buthionine sulfoximine (BSO) or LPS. LPS induces its inflammatory actions in macrophages via TLR4 while BSO is an inhibitor of the glutamatecysteinyl ligase (GCL) enzyme and thus may exhibit a "pro-oxidative" action by depleting glutathione pools. Didox inhibited both BSO and LPS-mediated intracellular \( \text{H}_2\text{O}_2 \) production and this was demonstrated via reduction of dichlorofluorescein (DCF) fluorescence in Didox-treated Raw 264.7 cells. Accumulation of DCF indicates the production of \( \text{H}_2\text{O}_2 \), a redox-active molecule (Bass et al., 1983, Royall and Ischiropoulos, 1993). Reduction of fluorescence therefore suggests that Didox inhibits \( \text{H}_2\text{O}_2 \) generation, an event that directly correlates with upregulation of catalase mRNA expression and could suggest that Didox reduces \( \text{H}_2\text{O}_2 \) levels either by upregulating catalase gene expression or activity. Didox upregulates Gpx mRNA levels in Raw 264.7 cells and Gpx could also be responsible for reduced \( \text{H}_2\text{O}_2 \) levels as it also catalyses the scavenging and decomposition of \( \text{H}_2\text{O}_2 \) in these cells.

Since SOD mRNA levels were upregulated in Didox-treated Raw 264.7 cells and SOD dismantles superoxide (\( \text{O}_2^• \)) radicals, it was necessary to assess the production of intracellular \( \text{O}_2^• \) levels in LPS-activated Raw cells after treatment with Didox. Superoxide produced in cells was measured using the cell-


permeable dye dihydroethidium (DHE), which mostly binds to nuclear DNA when oxidized by superoxide and emits red fluorescence whereas the unoxidized DHE emits blue fluorescence and remains localized mostly in the cytoplasm. DHE has been specifically recommended for assessments of superoxide production in cells because of its relatively high specificity (Zhao et al., 2003). A vigorous red fluorescence was observed in LPS-treated Raw cells while Didox treatment significantly reduced the red fluorescence during 24h incubation. Further addition of BSO, which depletes the GSH pools, exacerbated and increased superoxide mediated fluorescence especially when this was combined with LPS treatment of Raw cells. Didox rescued both BSO and LPS induced superoxide production as shown by the resulting diminished red fluorescence post-treatment. Potential implications of this reduction in intracellular detection and imaging of superoxide by Didox in activated Raw cells is of significant importance since superoxide radicals represent a highly reactive and cytotoxic group of free radicals contributing to the generation of oxidative stress in inflammatory diseases.

Several lines of evidence have revealed a requirement of ROS generation in NF-κB activation and suggest that ROS production leads to site-directed NF-κB oxidation resulting in increased NF-κB activation (Jaramillo et al., 2005, Jaramillo and Olivier, 2002). Previous studies have also demonstrated that certain antioxidants may block translocation of activated NF-κB complexes from the cytoplasm to the nucleus, thus inhibiting NF-κB mediated gene activation (Bowie
et al., 1997). LPS-stimulation of Raw cells leads to phosphorylation of NF-κB dimers (p65/p50) in the cytoplasm. Upon phosphorylation, the p65/p50 dimer translocates from the cytoplasm to the nucleus and activates expression of target inflammatory genes (including IL-6, TNF-α, iNOS, Cox-2) by binding to specific promoter consensus sequences. We hypothesized that Didox-mediated inhibition of LPS-induced inflammation in Raw 264.7 murine macrophages may be effected via modifications on NF-κB activity or function. In order to test this hypothesis, we examined nuclear localization of NF-κB complexes in Raw 264.7 murine macrophages after LPS or Didox treatment using immunofluorescence microscopy.

LPS stimulation of Raw 264.7 murine macrophages resulted in prompt translocation of p65 from the cytoplasm to the nucleus. However, Didox treatment counteracted this LPS-mediated p65 translocation since more p65 was observed to be present mostly in the cytoplasm than in the nucleus of these macrophages. The Didox-mediated inhibition of NF-κB translocation was confirmed using a Western blot analysis which revealed higher p65 protein levels in the nucleus after LPS-stimulation of Raw cells, whereas addition of Didox lowered this LPS-induced nuclear p65 protein expression. This inhibition of NF-κB nuclear translocation suggests an ability of Didox to suppress oxidative stress induction in Raw 264.7 murine macrophages by inhibiting the NF-κB pathway.
To determine the effect of Didox on Raw 264.7 murine macrophage functionality a phagocytosis assay was carried out using FITC-labeled latex beads and TRITC-labeled yeasts. To achieve this, we incubated Raw macrophages with FITC-labeled latex beads in the presence of either LPS or Didox or both and assessed the uptake of beads using immunofluorescence microscopy. Non-engulfed excess FITC-beads were washed and quenched with trypan blue to ensure that the fluorescence observed only reflects those beads internalized by macrophages. Most beads were internalized by macrophages after stimulation with LPS alone but addition of Didox reduced this LPS-mediated bead uptake. Although staining of F-actin in Raw 264.7 cells revealed that the actin cytoskeletal reorganization was robust and extensive with LPS treatment and vigorous extensions and nodules were observed in the absence of Didox than when Didox was added, the influence of Didox on the actin cytoskeleton was not strong enough to qualify it as a significant inhibition. There could be other signaling events involved in the reorganization of F-actin since the effect of Didox on the actin network at microscopic level is too subtle to make a clear conclusive assessment.

**CONCLUSION**

In summary, we found that Didox inhibited LPS-induced oxidative stress, ROS generation, IL-6 production, NO production, phagocytic activity, and NF-κB nuclear translocation in Raw 264.7 murine macrophages. Due to its antioxidant
characteristics, Didox might be used to prevent ROS-induced inflammatory diseases such as sepsis-related injuries, cancer, fibrosis, emphysema, and arthritis. More studies are needed to demonstrate its mechanism of action and potential benefits in humans, however. Other studies done with clinical trials on patients with advanced breast cancer treated with Didox at 6 g m\(^{-2}\) given by intravenous infusion over 36 h every 3 weeks demonstrated minimal toxicity with high drug clearance (Carmichael et al., 1990, Rubens et al., 1991). This shows that Didox administration can be tolerated by patients with minimal side effects and once its use in inflammation-based diseases is delineated, its application in clinical settings can be achieved with relative rapidity.
CHAPTER 3:
DIDOX MODULATES AHR LIGAND-INDUCED OXIDATIVE STRESS, CYP1A1 INDUCTION, AND AHR-ACTIVITY IN RAW 264.7 MURINE MACROPHAGES.

ABSTRACT

Aryl hydrocarbon receptor (AHR) activation has been shown to induce oxidative stress, various transcription factors, and subsequent inflammatory processes mediating toxic and carcinogenic effects in vascular endothelial cells. Didox (3,4-Dihydroxybenzohydroxamine) is a powerful synthetic antioxidant and one of the most potent ribonucleotide reductase (RR) inhibitors, revealing an antitumor effect in several experimental studies. To test the hypothesis that Didox can modify AHR-mediated oxidative stress, Raw 264.7 murine macrophage cells were treated with AHR ligands (PCB126, E804, IO) with or without Didox for 24h. Didox strongly inhibited oxidative stress induced by PCB126 as measured by DCF fluorescence.

The role of cytochrome P450 1A1 (CYP1A1) in the AHR ligand-induced toxicity was investigated. Didox at 100 µM concentrations markedly inhibited CYP1A1 and AHR mRNA levels. Using immunofluorescence microscopy, Didox was shown to significantly inhibit both AHR nuclear translocation and CYP1A1 expression in Raw 264.7 cells treated with these AHR ligands. Furthermore, Didox downregulated reactive oxygen species production induced by PCB126, E804, and IO in Raw 264.7 murine macrophages. These data suggest that
oxidative stress regulatory effects of Didox may be useful in managing inflammatory diseases initiated by exposure to AHR activating xenobiotics.

**INTRODUCTION**

Exposure to toxic polycyclic aromatic hydrocarbons produces oxidative stress and raises a number of toxic and carcinogenic responses in experimental animals and humans mediated for the most part by the aryl hydrocarbon – or dioxin – receptor (AHR) (Meijer *et al.*, 2007). The AHR is a ligand-activated transcription factor whose central role in the induction of drug-metabolizing enzymes has long been recognized. For quite some time now, it has become clear that the AHR also functions in pathways outside of its role in detoxification and that perturbation of these pathways by xenobiotic ligands may be an important part of the toxicity of these compounds. The aryl hydrocarbon (dioxin) receptor (AHR) is a cytosolic ligand-activated transcription factor that mediates many toxic and carcinogenic effects in animals and possibly in humans (Barouki *et al.*, 2007).

It is generally accepted that its activation in vertebrates causes the toxic and carcinogenic effects of a wide variety of environmental contaminants such as dioxin (TCDD, 2,3,7,8-tetrachlorodibenzo-p-dioxin), coplanar polychlorinated biphenyls (PCBs) and polycyclic or halogenated aromatic hydrocarbons (PAHs or HAHs). As a consequence of AHR activation, many detoxification genes are transcriptionally induced, including those coding for the Phase I xenobiotic-
metabolizing cytochrome P450 enzymes CYP1A1, CYP1A2, CYP1B1, and CYP2S1, and the phase II enzymes UDP-glucuronosyl transferase UGT1A6, NAD(P)H-dependent quinone oxydoreductase-1 NQO1, the aldehyde dehydrogenase ALDH3A1, and several glutathione-S-transferases (Barouki et al., 2007).

AHR is a member of the bHLH/PAS family of heterodimeric transcriptional regulators (basic-region helix-loop-helix/Period [PER]-Aryl hydrocarbon receptor nuclear translocator [ARNT]-single minded [SIM]) (Hoffman et al., 1991) involved in regulation of development and in control of circadian rhythm, neurogenesis, metabolism and stress response to hypoxia. Evidence from AHR knockout mice, however, points to functions of the receptor beyond xenobiotic metabolism at several physiologic roles that may contribute to the toxic response. Ablation of the Ahr gene in mice leads to cardiovascular disease, hepatic fibrosis, reduced liver size, spleen T-cell deficiency, dermal fibrosis, liver retinoid accumulation and shortening of life span (reviewed in Barouki et al., 2007), suggesting that it has biological functions other than xenobiotic detoxification that likely contribute to the overall toxic response resulting from its activation.

The AHR is widely expressed in practically all mouse tissues (Abbott et al., 1995), and in humans expression is high in lung, thymus, kidney and liver. In the absence of ligand, the AHR exists as part of a cytosolic protein complex containing two HSP90 chaperone molecules, the HSP90-interacting protein p23
and the immunophilin-like protein XAP2 (Petrulis et al., 2003). Activation by ligand is followed by translocation of the complex into the nucleus, dissociation from the chaperone proteins and heterodimerization with ARNT. This AHR-ARNT heterodimer interacts with several histone acetyltransferases and chromatin remodeling factors and the resulting complex binds to consensus regulatory sequences termed AHREs (aryl hydrocarbon response elements; also XREs or DREs), located in the promoters of target genes, and by mechanisms not yet well characterized, recruits RNA polymerase II to initiate transcription. The activated AHR is quickly exported to the cytosol where it is degraded by the 26S proteasome (Pollenz, 2002), hence preventing constitutive receptor activity.

Activation of the AHR by high-affinity HAH or PAH ligands results in a wide range of cell cycle perturbations, including G₀/G₁ and G₂/M arrest, diminished capacity for DNA replication, and inhibition of cell proliferation. These alternative functions of the AHR are often accomplished in the absence of an exogenous ligand, but the underlying molecular mechanisms governing these processes remain elusive in part because no definitive endogenous ligands have been identified (reviewed in Puka et al., 2002). At present, all available evidence indicates that the AHR can trigger signal transduction pathways involved in proliferation, differentiation or apoptosis by mechanisms dependent on xenobiotic ligands or on endogenous activities that may be ligand mediated or completely ligand independent. These functions of the AHR coexist with its well-characterized toxicological functions.
involving the induction of Phase I and Phase II genes for the detoxification of foreign compounds.

Polychlorinated biphenyls (PCBs) are widespread persistent residual environmental contaminants and possess the ability to activate the AHR pathway via binding to AHR. Some of the PCBs, e.g. 3,3',4,4',5-pentachlorinated biphenyls 126 (PCB126), have been demonstrated to produce extensive cellular oxidative stress in vertebrates, including rodents (Fadhel et al., 2002) and human hepatoma cells (Park et al., 1996). The antioxidant vitamin E has been shown to have a protective effect against the gross morphological changes induced by PCB126 exposure in zebrafish embryos (Na et al., 2009), indicating a potential use of similar antioxidants in relieving oxidative stress induced by PCBs. PCB-126 has been also shown to potentiate enhancement of plasma lysozyme levels and increase Cyp1A1 induction in channel catfish, Ictalurus punctatus (Burton et al., 2002; Rice and Roszell, 1998). The co-planar structure of PCB 126 allows it to interact with AHR and activate subsequent AHR nuclear translocation.

Other molecules discovered to possess ability to bind AHR include indirubin and indirubin-derivatives (Kawanishi et al., 2003). Indirubin derivatives such as Indirubin-3’-(2,3 dihydroxypropyl)-oximether (E804) have been demonstrated to block Stat3 signaling in human breast and prostate cancer cells via interactions with AHR. Studies on AHR activation using indirubin derivatives demonstrated...
that although derivatives such as 6-Bromoindirubin-3′-monoxime (BIO) and 5-Iodo-Indirubin-3′-monoxime (5-Iodo) bind to AHR and induce its nuclear translocation, they also triggered ARNT-independent nuclear redistribution of AHR (Knockaert et al., 2004). Indirubin-3-monoxime is an aryl hydrocarbon receptor (AHR)-binding, idole-derived compound known to be a potent inducer of CYP1A1, at least in hepatic cell lines and indirubin-3′-monoxime has been demonstrated to either act as classical AHR ligand, or modulates endogenous AhR-regulated activity in U937 histiocytic lymphoma cells (Springs and Rice, 2006).

These results demonstrate that indirubins and indirubin derivatives are potent and functional ligands for AHR in living cells and that ARNT is not necessary for indirubin-induced nuclear translocation of AHR. The discovery of the interaction of indirubins with AHR opens the possibility that indirubins and indirubin derivatives prevent cell proliferation and induce cellular oxidative stress via the AHR pathway.

The persistence and prevalence of various exogenous AHR ligands in environmental resources necessitates a search for molecules that can inhibit AHR-mediated oxidative stress in an attempt to ameliorate AHR-induced disease conditions outlined above. Although an AHR-mediated oxidative stress response does not necessarily result in toxicity, it is a component in the mechanism of
many toxic events that generate high amounts of ROS and RNS which ultimately result in cellular stress, cell death or carcinogenesis. This is because AHR regulates the expression of phase I and phase II detoxification enzymes that, by the nature of their enzymatic activities, generate electrophilic reaction, which cause oxidative stress, and conjugation reactions which attempt to combat it. Therefore AHR activation results in a shift in the cellular redox balance, suggesting a diverse and sophisticated phenomenon in oxidative stress response and generation.

Since Vitamin E has shown potential oxidative stress inhibition capacity in PCB126 induced AHR activation, similar antioxidants and NSAIDS such as Didox can be evaluated for their ability to curb AHR-mediated oxidative stress. The absence of endogenous ligands to AHR opens possibilities that AHR inhibitors may be able to associate with AHR in various ways since endogenous ligands may be using similar associations themselves. Didox is presented here as such a potential AHR pathway inhibitor due to its well documented ability to scavenge free radicals, block production of inflammatory cytokines, and reduce oxidative stress in various experimental modules without inducing major side effects and cytotoxicity. Its structural outlook may not permit binding to AHR but its activity profiles may allow it to associate with AHR mediators and the AHR pathway in various ways when acting as an antioxidant agent.
MATERIALS AND METHODS

Materials

Indirubin derivatives and PCBs were obtained as follows: IO or Indirubin-3'-monoxime (A.G. Scientific Inc., San Diego, CA), E804 or Indirubin-3'-(2,3 dihydroxypropyl) oximether (Alexis Biochemical, San Diego, CA), PCB126 or 3,3',4,4',5-pentachlorobiphenyl (Ultra Scientific, North Kingston, RI), and PCB104 or 2,4,6,2',6'-pentachlorobiphenyl (Ultra Scientific, North Kingston, RI). Didox was provided by Dr. Howard Elford, Molecules for Health Inc. (Richmond, VA) and phorbol-12-mristate-13-acetate (PMA) was purchased from A.G. Scientific Inc., San Diego, CA. LPS from E. coli serotype R515(Re) (ultra pure, TLR4grade™) was purchased from Alexis biochemicals, San Diego, CA and FBS from Atlas biological, Fort Collins, CO. DMEM, FBS, HEPES, glucose, sodium bicarbonate, penicillin, streptomycin, paraformaldehyde, Triton X-100, sodium borohydride, buthionine sulfoximine and dimethyl sulfoxide (DMSO Hybrid Max®) were purchased from Sigma, St Louis, MO.

H$_2$DCF, DHE, DAF-2 DA (Molecular Probes, Eugene, OR), rabbit anti-AHR polyclonal antibody (Thermo Fisher Sci., Fremont, CA), goat-anti rabbit polyclonal Ab, Raw 264.7 murine macrophage cells (ATCC®, Manassas, VA), RT$^2$ Profiler™ PCR Array System, RT$^2$ First Strand Kit and RT$^2$ qPCR Master Mix were purchased from SABiosciences Corp., Frederick, MD, PCR primers from IDT®, Coralville, IA, TRI-reagent® from Molecular Research Center,
Inc. Cincinnati, OH and Halt™ Protease Inhibitor Cocktail from Thermo Fisher Sci., Rockford, IL. All other chemicals were purchased from Sigma, St Louis, MO.

**Cell Cultures**

Murine macrophage RAW 264.7 cells were cultured in Dulbecco’s modified Eagle’s medium (DMEM) containing 25 mM HEPES buffer, 3 g/L D-glucose, 1.5 g/L sodium bicarbonate and 4 mM L-glutamine. The culture media were supplemented with 10% fetal bovine serum, penicillin (100 units/ml) and streptomycin (100 µg/ml). All cultures were maintained in a humidified incubator in 5% CO₂ at 37 °C and cells were be split for sub-culturing two times a week.

**Treatment Preparations**

Didox (3,4-Dihydroxybenzohydroxamine) obtained was dissolved in dimethyl sulfoxide (DMSO Hybri Max® purchased from Sigma, St Louis, MO) to a final stock concentration of 200 mM and further diluted in culture medium to the working serially diluted concentrations ranging from 3.12 – 200 µM. LPS (E. coli, Serotype R515 ultra-pure, TLR4 grade™ purchased from Alexis Biochemicals, San Diego, CA) was diluted in serum-free culture medium before adding to culture treatments. Indirubin derivatives and PCBs were also dissolved in DMSO as stock solutions of 10⁻² M.
Flow Cytometry ApoScreen™ Annexin V Apoptosis Assay

Assessment of ability of AHR ligands, Didox or LPS to induced apoptosis in Raw cells was carried out using AnnexinV/PI double staining. Apoptosis was quantified by measurement of externalized phosphatidylserine residues as detected using ApoScreen™ Annexin V Apoptosis Kit (Southern Biotech, AL, USA). Raw 264.7 murine macrophages were seeded at 2.5 x 10^6 cells/well in 24-well plates for 3hrs and treated with 10 µM of AHR ligands (PCB126, E804, IO) or non-AHR ligands (PCB104) with or without Didox (100µM) for 24hrs. After treatment cells were washed with ice-cold PBS and 500 µl of Annexin V binding buffer. The binding buffer (100 µl) was replaced in all wells, 5 µl of Annexin V-FITC was added, and the mixture was incubated for 15 min on ice with slow shaker in the dark. Without washing, 380 µL of cold 1X binding buffer and 10 uL of PI were added to each tube. After washing the samples, aliquots were processed by flow cytometer using FACScanTM (BD Biosciences, San Jose, CA) and 10,000 cells were scored in each event.

Fluorescence Microscopy Analysis of Cell death using Hoescht/PI staining

Cells were treated as indicated above on Lab-Tek® 8-chamber glass cultures slides (Nalge Nunc Int., IL, USA) or Corning glass cover slips and Hoescht/PI stain was added the cells for 15min. After washing off the dyes cells were mounted using 50% glycerol (in PBS) with colorless nail polish sealing and viewed under a fluorescent microscope.
Mouse Oxidative Stress Gene Analysis using RT² Profiler™ PCR-Arrays.

To search for candidate genes modulated by Didox after treatment of Raw cells with PCB126, cells were seeded at 5 X 10⁶ cells/ml in 75 cm² culture flasks (Corning Inc., NY) and allowed to adhere to flask surfaces to 3h. Cells were then treated with PCB126 in the presence or absence of Didox (100 µM) and RNA was extracted from cells after 24h using TRI-reagent® from Molecular Research Center, Inc. (Cincinnati, OH) and retrotranscribed by RT-PCR first strand kit (SuperArray Bioscience Corporation, Frederick, MD). Real-time PCR was performed using the Mouse Oxidative Stress RT² Profiler PCR Arrays and RT² Real-Timer SyBR Green/qPCR Master Mix from SuperArray Bioscience Corporation (Frederick, MD) on BioRad iQ5 real-time PCR detection system (Applied Biosystems). For data analysis the ∆∆Ct method was used; for each gene fold-changes were calculated as difference in gene expression between untreated controls and treated cell cultures. A positive value indicates gene up-regulation and a negative value indicates gene down-regulation.

AHR and Cyp1A1 Real-Time PCR assays

To evaluate the effect of AHR ligands (PCB126, E804, IO) on AHR and Cyp1A1 mRNA expression, RNA was extracted and cDNA obtained from treated murine macrophages as mentioned above. mRNA levels were analysed by using the following conditions: 1 cycle at 95°C for 10 min, 40 cycles at 95°C for 15 sec, 55°C for 1 min and quantified using primers listed in Table 3.1 below. The
quantities of these mRNAs were expressed as fold-changes compared to the untreated Raw 264.7 murine macrophage controls.

Table 3.1: Primer sequences used for RT-PCR validation of AHR and Cyp1A1 gene expression in Raw 264.7 macrophages after treatment with AHR ligands.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Sequence</th>
</tr>
</thead>
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<td>AHR</td>
<td>F: 5'-CTTTGCTGAACTCGGCTTGC-3' R: 5'-TTGCTGGGGGACACACCCTC-3'</td>
</tr>
<tr>
<td>CYP1A1</td>
<td>F: 5'-CCTCATGTACCTGGTAACCA-3' R: 5'-AAGGATGAATGCCGGAAGGT-3'</td>
</tr>
<tr>
<td>β-actin</td>
<td>F: 5'-TCATGAAGTGTGACGTTGACATCCGT-3' R: 5'-CCTAGAAGCATTTGCCTGACGATG-3'</td>
</tr>
</tbody>
</table>
Evaluation of AHR nuclear translocation using Immunofluorescence Microscopy.

To assess AHR activity in Raw 264.7 murine macrophages, immunofluorescence microscopy was used to monitor translocation of AHR from cytosol to nucleus after potentially binding to AHR ligands. Raw 264.7 cells were cultured cells in 8-chamber slides at 2 x10^5 cells/well and seeded for 3hrs followed by a 4h treatment with 10 µM of AHR ligands (PCB126, E804, IO) or non-AHR ligands (PCB104) with or without Didox (100µM). Cells were then fixed with 4% p-formaldehyde for 1hr and permeablized with 0.1% Triton X-100/1%BSA in PBS for 30 min on a slow shaker. To quench out any residual fluorescence, cells were treated with 0.01% sodium borohydride (in PBS) for 5 min and then incubated with 1% BSA for 30 min to block non-specific binding sites. Cells were then incubated for 1hr with rabbit anti-mouse AHR antibody followed by 1hr incubation with FITC-labeled goat anti-rabbit IgG. The nuclei were stained with DAPI addition for 5 minutes and cells were mounted on slides with coverslips using 50% glycerol in PBS. After sealing the edges with nail polish slides were viewed under Nikon AZ100 epi-fluorescent microscope.

Assessment of CYP1A1 induction in Raw 264.7 cells using Immunofluorescence Microscopy.

To assess CYP1A1 in Raw 264.7 murine macrophages after treatment with AHR ligands, immunofluorescence microscopy was used. Raw 264.7 cells were cultured cells on coverslips at 2 x10^5 cells/well in 6-well culture plates and
seeded for 3hrs followed by a 4h treatment with 10 μM of AHR ligands (PCB126, E804, IO) or non-AHR ligands (PCB104) with or without Didox (100µM). Cells were then fixed with 4% ρ-formaldehyde for 1hr and permeabilized with 0.1% Triton X-100/1%BSA in PBS for 30 min on a slow shaker. To quench out any residual fluorescence, cells were treated with 0.01% sodium borohydride (in PBS) for 5 min and then incubated with 1% BSA for 30 min to block non-specific binding sites. Cells were then incubated for 1hr with rabbit anti-mouse CYP1A1 antibody followed by 1hr incubation with FITC-labeled goat anti-rabbit IgG. The nuclei were stained with DAPI addition for 5 minutes and cells were mounted on slides with coverslips using 50% glycerol in PBS. After sealing the edges with nail polish slides were viewed under Nikon AZ100 epi-fluorescent microscope.

**Evaluation of ROS production using fluorescent microscopy**

Cells were seeded at 5 × 10^5 cells/well in eight chamber cell culture slides for 3h and then treated with PCB126 (10 μM) in the presence or absence of Didox (100μM). After 24h incubation, H$_2$DCF solution was added at 10$^{-5}$ M to all wells and incubated for an additional 30 min at 37˚C. The slides were washed twice with PBS, fixed with 4% ρ-formaldehyde for 1hr and mounted using glycerol:PBS (1:1) solution. The cover slip was placed over the mounting medium and the slides were sealed using nail polish. The slides were viewed under the fluorescence microscope and images capture using the digital camera.
Assessment of intracellular superoxide radical production in Raw 264.7 cells

Superoxide production in Raw cells was assayed by using the oxidative fluorescent dye DHE. DHE is oxidized on reaction with superoxide to ethidium bromide, which mostly binds to DNA in the nucleus and fluoresces red. Cells were seeded on cover slips at 2 x 10^5 cells/well (in 6-well plates) for 3hrs. To quench out any residual fluorescence, cells were treated with 0.01% sodium borohydride (in PBS) for 5 min. After loading cells with 10 μM DHE for 15min, cells were treated for 120 min with 1 μM of AHR ligands (PCB126, E804, IO) or non-AHR ligands (PCB104) with or without Didox (100μM). Cells were fixed and mounted on slides and analyzed using the Nikon AZ100 epi-fluorescent microscope. The un-oxidized DHE remains blue (λ_{Ex:Em}=355\,\text{nm}:420\text{nm}) while the oxidized DHE fluoresces red (λ_{Ex:Em}=355\,\text{nm}:420\text{nm}). Images were captured using the NIS-Elements Viewer 3.0.

Statistical Analysis

All experiments are representative of three repeats carried out in triplicates and the error bars represent the standard deviation from the mean average. All data representations were analyzed using Graphpad PRISM 4.0 (Graphpad, San Diego, CA, USA) or Sigmaplot (SPSS). Treatment effects were analysed using the paired t-test and p<0.05 was considered to be statistically significant.
RESULTS

Flow Cytometry ApoScreen™ Annexin V Apoptosis Assay

An apoptosis assay was carried out using AnnexinV/PI double staining and flow cytometry to assess cytotoxic potential of AHR ligands (PCB126, E804, BIO, IO), Didox or LPS in Raw 264.7 murine macrophages. None of the cells groups treated with Didox, LPS or AHR ligands showed significant signs of cell death or apoptosis (Fig 3.1). Cells which stained positive Annexin-V/PI were generally less than 1% in all cases investigated. Thus, almost all treated cells excluded PI stain and stained negative for Annexin V. The exclusion of PI and negative staining of Annexin-V allowed further exploration of action these drugs knowing that they are not cytotoxic to Raw cells at the concentrations tested.

Fluorescence Microscopy Analysis of Cell death using Hoescht/PI staining

Hoechst 33342, a type of blue-fluorescence dye (excitation/emission maxima $\approx 350/461$ nm when bound to DNA), stains the condensed chromatin in apoptotic cells more brightly than normal chromatin. Propidium iodide (PI), a red-fluorescence dye (excitation/emission maxima $\approx 535/617$ nm when bound to DNA), is only permeant to dead cells. The staining pattern resulting from the simultaneous use of these dyes makes it possible to distinguish normal, apoptotic, and dead cell populations by fluorescence microscopy. Cells were treated with Didox or AHR ligands and stained with Hoechst/PI double stain. Fluorescent microscope images confirmed that none of the drugs induced
apoptosis or cell death since all Raw 264.7 cells excluded PI stain and only accepted the Hoechst stain (Fig 3.2).
Figure 3.1. Flow cytometric analysis of Raw 264.7 cells treated with Didox, LPS and AHR-ligands (PCB126, E804, IO and BIO). Raw 264.7 murine macrophages were seeded in 24 well plates for 3hrs and treated with 10 µM of AHR ligands (PCB126, E804, IO), LPS (0.1 µg/ml) or Didox (100µM) for 24hrs. Apoptosis was quantified by measurement of externalized phosphatidylserine residues as detected by flow cytometer and 10 000 cells were scored in each event.
Figure 3.2. Hoechst/PI apoptosis evaluation of Raw 264.7 cells treated with AHR ligands (PCB126, E804, IO) and Didox. Cells were treated as indicated above, Hoechst/PI stain was added and cells were viewed under a fluorescent microscope after fixing and mounting on glass slide.
Evaluation of AHR nuclear translocation using Immunofluorescence Microscopy.

The induction AHR ligands mediated oxidative stress in many cells occurs via binding to the aryl hydrocarbon receptor (AHR) and activating the AHR pathway. The inactive form of the AHR molecule resides in the cytoplasm and upon binding to AHR ligands it translocates to the nucleus to induce targeted expression of responsive genes. To assess AHR nuclear translocation after binding AHR ligands (PCB126, E804, IO) in Raw 264.7 murine macrophages, immunofluorescence microscopy was used. All AHR ligands used were able to induce AHR nuclear translocation with PCB126 and E804 inducing higher levels of nuclear translocation as evidence by lesser AHR in cytoplasm and more fluorescence in the nucleus (Fig 3.3). The AHR ligands induced translocation was evidently suppressed by addition of Didox since lower nuclear AHR fluorescence was observed upon its addition. PCB104 is not an AHR ligand and was employed as a negative control to ensure specificity of AHR ligands to AHR binding.
Figure 3.3. The effect of Didox on PCB126, E804, and IO-induced AHR nuclear translocation in Raw 264.7 murine macrophages. Raw 264.7 cells were treated with AHR ligands in the presence or absence of Didox and immunofluorescence microscopy was used to monitor translocation of AHR from cytosol to nucleus (bars = 20 µm)
Assessment of CYP1A1 induction in Raw 264.7 cells using Immuno-fluorescence Microscopy.

Induction of Cyp1A1 is known to represent a significant event in the toxicity of AHR ligands and the Cyp1A1 gene provides a model system for studying the mechanism of gene transcription by AHR. Therefore expression of Cyp1A1 after AHR activation is indicative of activation of the AHR pathway and further confirms binding of AHR ligands to AHR. To assess CYP1A1 activity in Raw 264.7 murine macrophages after treatment with candidate AHR ligands (PCB126, E804, IO), immunofluorescence microscopy was used. PCB126 and E804 consistently induced higher Cyp1A1 expression as evidenced by higher fluorescence in Raw cells after treatment (Fig 3.4 and 3.4b).

IO also induced Cyp1A1 expression albeit at lower levels than PCB126 and E804. The expression of Cyp1A1 induced these AHR ligands was variably inhibited by addition of Didox and lower fluorescence was observed in the Raw cells when Didox was included in the regimen. PCB104 is not an AHR ligand and as expected did not induce Cyp1A1 expression in Raw cells.
Figure 3.4. The effect of Didox on CYP1A1 induction by AHR ligands (PCB126, E804, and IO) in Raw 264.7 murine macrophages. Raw 264.7 cells were cultured cells in 6-well culture plates and treated with 10 μM of AHR ligands (PCB126, E804, IO) or non-AHR ligands (PCB104) with or without Didox (100μM). CYP1A1 antibody was measure using FITC-labeled goat anti-rabbit IgG and nuclei were stained with DAPI. Slides were viewed under Nikon AZ100 Epi-Fluorescent Microscope.
Figure 3.4b. Quantitative analysis of the effect of Didox on CYP1A1 induction by AHR ligands (PCB126, E804, IO) in Raw 264.7 murine macrophages. Raw 264.7 cells were cultured cells in 6-well culture plates and treated with 10 µM of AHR ligands (PCB126, E804, IO) or non-AHR ligands (PCB104) with or without Didox (100 µM). CYP1A1 antibody was measured using FITC-labeled goat anti-rabbit IgG and nuclei were stained with DAPI. Slides were viewed under Nikon AZ100 Epi-Fluorescent Microscope. Mean fluorescence intensities were measured in various fields in triplicates and treatment effects were analysed using the paired t-test and p<0.05 (*) was considered to be statistically significant.
**AHR and Cyp1a1 Gene expression using Real-Time PCR**

Real-time PCR using specific AHR and CYP1A1 primers was carried out to evaluate the effect of AHR ligands (PCB126, E804, IO) on AHR and Cyp1A1 mRNA expression in Raw 264.7 murine macrophages. PCB126 induced the highest mRNA expression of more than 60-fold above the controls followed by E804 with a 24-fold change and IO with less than 10-fold difference (Fig 3.5). This AHR ligand-induced mRNA expression was significantly suppressed by addition of Didox. Thus, Didox inhibited mRNA expression levels of AHR and Cyp1A1 in Raw 264.7 murine macrophages treated with PCB126, E804 and IO.

**Mouse Oxidative Stress Gene Analysis using RT² Profiler™ PCR-Arrays.**

Once it was established that these AHR ligands (at 10 µM) do not induce cell death while inducing AHR and Cyp1A1 activity in Raw 264.7 cells, a search for oxidative stress related candidate genes was carried out using PCB126, a more potent AHR ligand. The Mouse Oxidative Stress RT² Profiler™ PCR-Arrays revealed that expression of several genes playing important roles in induction of oxidative stress in macrophages were significantly attenuated upon addition of PCB126 (Table 3.2). Of these, genes of the NADPH oxidase family were notably induced whereas most antioxidant genes such as catalase, glutathione peroxidases, peroxiredoxins and superoxide dismutases, were variably suppressed upon addition of PCB126 in Raw 264.7 cells. Addition of Didox suppressed PCB126-induced expression of NADPH oxidases while inducing
expression of antioxidant genes (Catalase, glutathione peroxidase, superoxide dismutase, and peroxiredoxins) in Raw 264.7 cells.

![Graph showing mRNA expression of AHR and CYP1A1 for DX/LPS and PCB126 treatments](image-url)
Figure 3.5. The effect of Didox and LPS on AHR and CYP1A1 mRNA expression in Raw 264.7 cells (Real-time PCR study with specific primers).

To evaluate the effect of AHR ligands (PCB126, E804, IO) on AHR and Cyp1a1 mRNA expression, RNA was extracted and cDNA obtained from treated murine macrophages to run a real-time PCR assay as mentioned in methods above.
Table 3.2. Real-time PCR multigene Array for evaluation of mouse oxidative stress related genes in Raw 264.7 cells after treatment with PCB126 in presence or absence of Didox. For data analysis the $\Delta\Delta C_t$ method was used; for each gene fold-changes were calculated as difference in gene expression between untreated controls and treated cell cultures. A positive value indicates gene up-regulation and a negative value indicates gene down-regulation.

<table>
<thead>
<tr>
<th>Gene name</th>
<th>Genbank No.</th>
<th>Symbol</th>
<th>DX</th>
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<tr>
<td>Glutathione peroxidase 1</td>
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**Didox inhibits PCB126-induced ROS production in Raw cells**

Intracellular ROS production in Raw 264.7 cells was measured using 5-(and-6)-chloromethyl-2',7'-dichlorodihydrofluorescein diacetate (CM-H$_2$DCFDA) which is hydrolyzed to DCFH in the cell, and DCFH is oxidized to form highly fluorescent DCF in the presence of the correct oxidant. ROS generation was detected as a result of DCFH oxidation after addition of PCB126 in Raw 264.7 cells and this was indicated by higher fluorescence in the cells upon treatment (Fig 3.6 and 3.6b). The resulting fluorescence was subdued by co-incubation with Didox to indicate suppression of PCB126-induced ROS production.
Figure 3.6. Didox inhibits ROS production in Raw 264.7 cells treated with PCB126.

Cells were seeded at $5 \times 10^5$ cells/well in eight chamber cell culture slides for 3h and then treated with PCB126 (10 µM) in the presence or absence of Didox (100µM). H$_2$DCF solution was added at $10^5$M to all wells and incubated for an additional 30 min at 37°C. The slides were viewed under the fluorescence microscope and images capture using the digital camera.
Figure 3.6b. Quantitative assessment of ROS production in Raw 264.7 cells treated with PCB126 and Didox. Cells were seeded at $5 \times 10^5$ cells/well in eight chamber cell culture slides for 3h and then treated with PCB126 (10 µM) in the presence or absence of Didox (100µM). $H_2$DCF solution was added at $10^{-5}$M to all wells and incubated for an additional 30 min at 37°C. The slides were viewed under the fluorescence microscope and images capture using the digital camera. Mean fluorescence intensities were measured in various fields in triplicates and treatment effects were analysed using the paired $t$-test and $p<0.05$ (*) was considered to be statistically significant.
Assessment of intracellular superoxide radical production in Raw 264.7 cells

Of the ROS molecules produced by macrophages during inflammation, superoxides are in the most reactive category. DHE, a vital dye sensitive to superoxide oxidation, was employed to detect superoxide production in Raw cells after treatment with PCB126 and E804 and to determine if this production can be attenuated by Didox. An intense red fluorescence was observed upon treatment of cells with PCB126 and E804 to indicate a robust production of intracellular superoxide radicals in Raw 264.7 cells (Fig 3.7 and 3.7b). This robust red fluorescence was significantly reduced by addition of Didox in these cells. The unoxidized DHE maintained a blue fluorescence in all culture treatments.
Figure 3.7. Didox inhibits PCB126 and E804-induced oxidative burst and intracellular superoxide production in Raw 264.7 cells. Cells were loaded with 10 µM DHE for 15min, and treated for 120 min with 10 µM of AHR ligands (PCB126 and E804) or non-AHR ligands (PCB104) with or without Didox (100µM). Slides were analyzed using the Nikon AZ100 Epi-Fluorescent Microscope (Bar = 20 µm).
Figure 3.7b. Quantitative analysis of oxidative burst and intracellular superoxide production in Raw 264.7 cells treated Didox, PCB126 and E804. Cells were loaded with 10 µM DHE for 15 min, and treated for 120 min with 10 µM of AHR ligands (PCB126 and E804) with or without Didox (100 µM). Slides were analyzed using the Nikon AZ100 Epi-Fluorescent Microscope. Mean fluorescence intensities were measured in various fields in triplicates and treatment effects were analysed using the paired t-test and *p<0.05 (*) was considered to be statistically significant (compared to control unless otherwise indicated).
DISCUSSION

The aryl hydrocarbon receptor (AHR) is a ligand activated transcription factor that regulates genes involved in xenobiotic metabolism, cell proliferation, and differentiation (Meijer et al., 2007). There have been many reports about toxic mechanisms of AHR ligands or xenobiotics capable of activating the AHR pathway and they demonstrated the relationship between oxidative stress and the site of target organs. Certain PCBs can compromise normal functions of vascular endothelial cells by activating oxidative stress-sensitive signaling pathways and subsequent proinflammatory events critical in the pathology of atherosclerosis and cardiovascular disease (Henning et al., 2002). However, there are some reports about the protective effects of some antioxidants against AHR ligands in cell culture systems with vitamin E being shown to protect against neurotoxicity caused by Aroclor1254 or PBC-induced toxicity if zebrafish embryos (Na et al., 2009).

Thus, it was worthwhile to investigate the ability of Didox to inhibit the AHR pathway in Raw 264.7 murine macrophages since it has been demonstrated to be a powerful antioxidant in various experimental models. Raw 264.7 murine macrophages were selected as a preferred model for this study since macrophages play an active role in oxidative stress mediated diseases and also express AHR along with associated molecules of the AHT pathway. Several AHR ligands were identified and used as candidates for use in the study, and this
include PCB126, E804, and IO whereas a non-AHR binding molecule, PCB104, was used to ensure specificity of reactions.

Since AHR ligands can induce lethal toxicity in macrophages, it was important to first evaluate the cytotoxic profiles of these candidate ligands and their ability to induce apoptosis in Raw 264.7 macrophages. Using the Annexin-V/PI flow cytometry assay and Hoechst/PI apoptosis assay, it is clearly demonstrated here that none of the AHR ligands induced apoptosis in these Raw macrophages when used at 10 µM concentration for 24h. More than 98% of the Raw 264.7 cells remained viable and showed no noticeable signs of apoptosis after treatment with PCB126, E804, or IO. Cells mostly stained negative for Annexin-V and excluded PI while staining positive for the cell permeant, Hoechst 33342. It was therefore concluded that these doses can be safely used for evaluating AHR pathway associated responses in these cells line for durations up to 24h without cell death induction.

The next step was to evaluate if these candidate xenobiotics can bind to AHR and induce its translocation from the cytosol to the nucleus. Previous studies using urine of healthy individuals as a starting biological material to identify AHR ligands recognized indigo and indirubins as potent AHR activators. Hence, indirubin derivatives such as E804 and IO were evaluated for the ability to bind AHR in Raw 264.7 murine macrophages. PCB126 has been well demonstrated to strongly activate AHR pathways in various developmental model systems and
its ability to activate AHR reactions in Raw 264.7 cells was also evaluated. Evaluation of AHR nuclear translocation using immunofluorescence microscopy showed that all the AHR ligands tested were able to induce movement of AHR from cytosol to the nucleus. PCB126 and E804 were shown to be more potent AHR activators compared to IO. Addition of Didox to these treatments resulted in lower AHR nuclear translocation and this indicated that Didox suppressed AHR activation mediated by PCB126, E804, and IO. PCB104 failed to induce nuclear translocation of AHR and this demonstrated the AHR binding specificity of the AHR ligands used. The absence of an identifiable endogenous AHR ligand has led to possibilities that AHR activation can be activated via association to AHR in ways other than direct AHR binding. To this effect, an antioxidant such as vitamin E has been subsequently shown to protect against gross morphological changes induced by PCB126 (Meijer et al., 2007). It is therefore possible that an antioxidant like Didox could be using related inhibitory features to suppress AHR binding of PCB126, E804, and IO in Raw 264.7 cells.

Activation of AHR leads to enhanced expression of a variety of genes and the most prominent of these is cytochrome P450 (CYP1A1), playing a major role in generating subsequent oxidative stress responses (Knockaert et al., 2004). Since PCB126, E804, and IO were shown to bind AHR in Raw 264.7 cells their ability to induce CYP1A1 expression was assessed. Immunofluorescence microscopy analysis of Raw 264.7 cells treated with these AHR ligands revealed a higher expression of CYP1A1 in these cells compared to untreated and PCB104 treated
controls. A more pronounced CYP1A1 expression was noticeable in PCB126 and E804 treated cells compared to IO-treated samples. Interestingly, these results correlate positively with AHR binding studies which revealed that PCB126 and E804 induced higher AHR nuclear translocation in Raw 264.7 cells compared to those treated with IO. When Didox was added to the treatment regimen containing these AHR ligands, CYP1A1 expression was significantly suppressed. These results suggest an inhibitory role of Didox against AHR activation and subsequent CYP1A1 expression in Raw 264.7 murine macrophages. Gene expression studies using Real-time PCR revealed that Didox inhibited PCB126-, E804-, and IO-induced AHR and CYP1A1 mRNA expression in Raw 264.7 macrophages. Didox might therefore be inhibiting AHR activation via suppression of both mRNA and protein expression of AHR and CYP1A1.

AHR activation has been shown to be accompanied by high levels of oxidative stress and production of reactive oxygen species in various cell culture and animal systems. A search for oxidative stress response genes was carried out to identify genes that may targets for PCB126 induced activation in Raw 264.7 murine macrophages. A mouse oxidative stress real-time PCR array was carried to evaluate 84 oxidative stress and cytotoxicity related genes in Raw 264.7 murine macrophages. PCB126 strongly increased mRNA expression of NADPH oxidase 1 and 4, NADPH oxidase activator, NADPH organizer and Parkinson disease 7 gene in raw cells. NADPH oxidase of phagocytes plays a crucial role in host defense by generating an oxidative burst and producing reactive oxygen
species (ROS) that are intended to kill invading microbes (Rada et al., 2004). Didox addition, however, lowered PCB126-induced mRNA expression of these NADPH oxidase family members as indicated by lowered fold changes when Didox was included in the treatments. Moreover, Didox increased expression of genes such as catalase, glutathione peroxidase, peroxiredoxin and superoxide dismutase. Catalase play a central role in the decomposition of hydrogen peroxide and, together with glutathione peroxidases and peroxiredoxins, form the highly efficient machinery for detoxifying cells by reducing hydrogen peroxide levels. Superoxide dismutases are very efficient at reducing the levels of highly reactive superoxide free radical. The increase in mRNA expression of this family of genes by Didox suggests an important role of Didox as an antioxidant molecule in Raw 264.7 murine macrophages against PCB126.

Due to the changes observed in profiles of antioxidant genes as effect by Didox, it therefore was necessary to further evaluate intracellular ROS production and reactive oxidative burst in Raw 264.7 after treatment with AHR ligands. ROS generation immunofluorescence assay using oxidation of DCFH to DCF revealed an increased intracellular ROS production in Raw 264.7 cells treated with PCB126, a more potent AHR activator. This PCB126-mediated ROS production was lowered by addition Didox. The reduction of PCB126-induced ROS production by Didox correlates with earlier RT-PCR gene expression which showed increased catalase expression. Hydrogen peroxide is mainly responsible for ROS production detected through DCFH system and since it is decomposed
by catalase-mediated catalysis, Didox-upregulation of catalase gene expression could be mainly responsible for the observed ROS suppression in Raw 264.7 macrophages.

Assessment of superoxide radical production in Raw 264.7 murine macrophages treated with AHR ligands was achieved using the DHE system which is highly sensitive to superoxide oxidation and emits a strong red fluorescence upon contact. A robust red fluorescence indicative of oxidative burst and increased superoxide production was observed when cells were treated with PCB126 and E804. IO also increased superoxide production compared to untreated and PCB104 controls. Intracellular superoxide production was strongly reduced by addition Didox in all treatments of Raw macrophages with AHR ligands. These results were however not surprising since Didox was earlier shown to increase expression of superoxide dismutase, an enzyme responsible for neutralizing superoxide radicals.
CONCLUSION

Dysfunction and aberrant activation of macrophages is a critical underlying cause of the initiation of cardiovascular, neurodegenerative and inflammatory diseases. In addition to macrophage dysfunction, another functional change in cardiovascular and neurodegenerative diseases is the activation of the endothelium that is manifested as an increase in the production of oxidative stress related molecules. These high levels of reactive oxygen molecules, along with cytokines and adhesion molecules, are proposed to mediate the inflammatory aspects of degenerative disease by regulating the entry of leukocytes, both macrophages and lymphocytes, into the vascular wall.

There is evidence that the CYP1A1 subfamily is present in most and possibly all animal and human systems, including macrophages. For example, CYP1A1 has been detected in endothelial linings of fish and rodents, as well as in cultured human and porcine endothelial cells (Smolowitz et al., 1992; Stegeman et al., 1995; Toborek et al., 1995; Celander et al., 1997; Zhao et al., 1998; Hennig et al., 1999; Annas et al., 2000; Schlezinger and Stegeman, 2001). Oxidative stress and increased CYP1A1 expression can be induced via There is increasing evidence that exposure to polycyclic aromatic hydrocarbons can lead to cardiovascular toxicity and atherosclerosis. For example, there was a significant increase in mortality from cardiovascular diseases among Swedish capacitor manufacturing workers exposed to PCBs for at least 5 years (Gustavsson and Hogstedt, 1997), and most excess deaths were due to cardiovascular disease in
power workers exposed to phenoxy herbicides and PCBs in waste transformer oil (Hay and Tarrel, 1997).

The ability of Didox to downregulate AHR-mediated activation therefore presents an antioxidant system that can reduce AHR-mediated oxidative stress and cellular toxicity in macrophages. Didox has also been shown to potently inhibit NF-κB activation in various experimental models. Earlier work by Henning’s Lab indicates that coplanar PCBs that function as AhR agonists may be proinflammatory by activating NF-κB in vascular endothelial cells (Henning et al., 2002) and that this resultant oxidative stress can play a critical role in observed PCB-mediated endothelial cell dysfunction (Hennig et al., 1996). The ability of Didox to inhibit NF-κB and AHR activation provides a non-steroid anti-inflammatory drug that may be used as a potential treatment in disease conditions mediated by polycyclic aromatic aromatic hydrocarbons.
OVERALL CONCLUSION

In this study, Didox has been demonstrated to block inflammation and oxidative stress induced by LPS and AHR ligands in Raw 264.7 murine macrophages. These results present Didox as a novel therapeutic compound that may block oxidative stress and treat inflammation-based diseases by regulating macrophage activity. The ability of Didox to inhibit oxidative stress and regulate macrophage activity compliments its ribonucleotide reductase inhibitory capacity. The multifaceted behavior of Didox arms it with numerous target point in treating ailments exacerbated by deregulated macrophage activation. This is because macrophages undergo a wide spectrum of polarized activation states and have the dual potential to elicit tissue destructive reactions and to promote tumor progression. In general, tumor-associated macrophages from established tumors and the related myeloid-derived suppressor cells help cancer progression and metastasis. Therefore, these types of destructive macrophages are a key component of pathways connecting inflammation and cancer. Didox has the ability to inhibit cancer growth due to its ribonucleotide reductase inhibitory potential and by demonstrating its ability to inhibit inflammation or suppress oxidative stress presents it with two important target points: cell division and inflammation.

In this study Didox has been demonstrated to inhibit production of TNF-α, Cox-2, NO, ROS, and IL-6. TNF-α and IL-6 are two of the major inflammatory cytokines targeted for therapeutic treatment of inflammation based disease conditions and the ability of Didox to suppress their production is of paramount importance. Cox-2 is an enzyme that mediates production of prostaglandins responsible for inflammation and pain. Cox-2 inhibitors have been sought after for decades to alleviate inflammation and pain caused by high Cox-2 expression. By exhibiting
Cox-2 inhibitory abilities, Didox provides a promising NSAID that can be used to block inflammation and pain associated with high levels of Cox-2. The most important aspect of Didox is its ability to upregulate gene expression of antioxidants such catalase, thioredoxins, superoxide dismutase, and glutathione peroxidase. This upregulation of antioxidants is most probably responsible for the reduction in ROS production observed upon treatment of LPS- or AHR-induced Raw 264.7 cells with Didox. NF-κB and AHR can be activated by presence of high reactive oxygen intermediates. Thus, the suppression of ROS production by Didox may be responsible for its inhibitory effect on NF-κB or AHR. NF-κB and AHR are redox sensors and by depleting the cellular ROS pool Didox might be indirectly suppressing NF-κB and AHR activation along with associated cytotoxicities. Further studies can be designed to determine if Didox directly binds NF-κB and AHR or whether their inhibition is through indirect association. Using an appropriate inflammation model system animal studies can be carried to determine if the inflammation and oxidative stress inhibitory potential of Didox shown in Raw 264.7 murine macrophages can be sustained in an in vivo system and to further determine if this drug carries other physiologically rewarding properties.

Chronic inflammation caused by aberrant macrophage activation and environmental contaminants such as those that activate the AHR pathway or NF-κB pathway play a pivotal role in pathogenesis of most diseases. Administered alone or in combination with other drugs that inhibit similar pathways, Didox provides a more effective candidate which can regulate oxidative stress and inflammation while boosting recovery and enhancing improved disease-free survival.
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