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3,4-Dihydroxy-Benzohydroxamic Acid (Diox) Suppresses Pro-inflammatory Profiles and Oxidative Stress in TLR4-Activated RAW264.7 Murine Macrophages

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Abstract

Diox (3,4-dihydroxy-benzohydroxamic acid), is a synthetic ribonucleotide reductase (RR) inhibitor derived from polyhydroxy-substituted benzohydroxamic acid, and originally developed as an anti-cancer agent. Some studies indicate that diox may have anti-oxidative stress-like properties, while other studies hint that diox may have anti-inflammatory properties. Using nitric oxide production in response to LPS treatment as a sensitive screening assay for anti-inflammatory compounds, we show that diox is very potent at levels as low as 6.25 μ M, with maximal inhibition at 100 μ M. A qRT-PCR array was then employed to screen diox for other potential anti-inflammatory and anti-oxidative stress-related properties. Diox was very potent in suppressing the expression of these arrayed mRNA in response to LPS, and in some cases diox alone suppressed expression. Using qRT-PCR as a follow up to the array, we demonstrated that diox suppresses LPS-induced mRNA levels of iNOS, IL-6, IL-1, TNF- α , NF- κ B (p65), and p38- α , after 24 h of treatment. Treatment with diox also suppresses the secretion of nitric oxide, IL-6, and IL-10. Furthermore, oxidative stress, as quantified by intracellular ROS levels in response to macrophage activators LPS and phorbol ester (PMA), and the glutathione depleting agent BSO, is reduced by treatment with diox. Moreover, we demonstrate that nuclear translocation of NF- κ B (p65) in response to LPS is inhibited by diox. These findings were supported by qRT-PCR for oxidative stress genes SOD1 and catalase. Overall, this study supports the conclusion that diox may have a future role in managing acute and chronic inflammatory diseases and oxidative stress due to high production of ROS.

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Keywords

RAW264.7 cells; TLR-4; Didox; oxidative stress; inflammation; iNOS

1. Introduction

Inflammation is often considered to be a localized physiological response to tissue damage, pathogen entry into extravascular spaces, and release of cellular debris from disrupted tissue integrity and necrosis [1, 2]. From a classical perspective, such responses, including recruitment of granulocytes from microvasculature, exudate accumulation, and increased local blood supply, are normal aspects of wound healing that ultimately lead to wound resolution and restoration to healthy tissues. Chronic inflammation, on the other hand, may lead to tissue destruction and loss of normal function associated with inflamed tissues, as can be seen in rheumatoid arthritis for example. More subtle forms of chronic inflammation are now ascribed to broadly defined disease states such as obesity [3, 4], atherosclerosis [5], Type II diabetes [3], and some forms of cancer [6, 7].

Because of their wide tissue distribution, macrophages are strategically located to provide an immediate defense against not only pathogens, but damaged tissues as well, which can lead to inflammatory responses to endogenous danger signals [2, 8, 9]. Macrophages, therefore, are a logical target for therapeutic approaches to treating chronic inflammation and oxidative stress associated with inflammation [10], and especially if macrophages can be polarized at will using non-toxic pharmacological interventions. With regard to anti-inflammatory agents, glucocorticoids and non-steroidal anti-inflammatory drugs (NSAID) are the most widely prescribed, yet both have negative long-terms side effects. For example, many glucocorticoids inhibit pro-inflammatory transcription factors, like NF- κ B, AP-1, and SMAD3, but also may disrupt the hypothalamic-pituitary-adrenal axis [11]. NSAIDs, such as aspirin and selective COX-2 inhibitors (e.g., Celecoxib) are effective, but come with side effects, including gastrointestinal bleeding with aspirin and negative cardiovascular effects with COX-2 inhibitors at high doses and long term treatment [12–14] Clearly, alternative pharmacological treatment approaches are needed.

Didox (3,4-dihydroxy-benzohydroxamic acid), is a synthetic ribonucleotide reductase (RR) inhibitor derived from polyhydroxy-substituted benzohydroxamic acid [15, 16]. This compound was originally developed as alternative to hydroxyurea, a potent, yet toxic ribonucleotide reductase inhibitor commonly used to treat sickle-cell disease, chronic myelogenous leukemia, mastocytosis, and other disorders [17–21]. As an iron scavenger, didox inhibits RR through iron deprivation, as iron is a cofactor for the R2-subunit of RR needed for generation and stabilization of free tyrosyl radicals [22]. Through the inhibition of RR, didox increases the radiosensitivity of cancer cells, resulting in a reduction of bcl-2 mediated resistance to apoptosis [23]. Earlier studies showed promise for didox as an antineoplastic agent [24–26], and is well tolerated in human patients [27, 28]. Subsequent to earlier studies showing inhibition of RR, other studies show that didox reduces the level of oxidative injury markers in brains of HIV patients with dementia [29], and thus may have potent anti-oxidative stress properties. To that end, other studies show that didox may inhibit NF- κ B activation [30], one of the major players in inflammation involving oxidative stress

[7, 31]. Furthermore, didox, and its chemically related congener trimidox, also inhibit T-cell proliferation in murine model of organ rejection and graft-vs-host disease, with concomitant effects on both pro-inflammatory and regulatory cytokines [23]. Taken together, these observations suggest that didox may have not only anti-neoplastic properties as originally intended, but anti-inflammatory and anti-oxidative stress properties as well.

The goal of this study was to determine if didox has anti-inflammatory and anti-oxidative stress-like properties in a simple *in vitro* model of LPS-induced pro-inflammatory profiles and oxidative stress. The RAW264.7 cell line is a common model for understanding the physiology of macrophages [32, 33], and is a routine *in vitro* model in immunopharmacology and immunotoxicology [34–36]. A specific aim of the study was to determine the effects of didox on aspects of inflammation and oxidative stress mediated through TLR-4 -induced NF- κ B signaling.

2.0 MATERIALS AND METHODS

2.1 Cells and cell culturing

The murine macrophage cell line RAW 264.7 was obtained from ATCC (Manassas, VA USA) and maintained in Dulbecco's modified Eagle's medium (DMEM; Cellgro) supplemented with 10% heat-inactivated bovine calf serum with iron (Hyclone #SH30072.03, Thermo Fisher), 20 mM HEPES, 10 mM L-glutamine, 100 U/mL penicillin, 100 μ g/mL streptomycin, 110 μ g/mL sodium pyruvate, 1% non-essential amino acids (100 X stock), 4.5 g/L glucose, and 1.5 g/L of NaCO₃, each from Sigma (Sigma Aldrich, St. Louis MO, USA). Cells were typically grown and maintained at 37°C with 5% CO₂ in Corning 75 cm² culture flasks.

2.2 Cytotoxicity assays

Didox was solubilized in DMSO (Sigma Aldrich) to a stock solution of 10⁻² M and stored at -20° C in sealed vials until ready for use. Stock preparations were diluted to a final working concentration using supplemented DMEM just prior to use. The chemical structure for didox is shown in Figure 1. Unless otherwise noted, cells were treated with Didox alone, with 0.1 μ g/mL LPS [*E. coli* serotype R515 (Re)(ultra-pure, TLR4grade™) from Alexis Biochemicals (San Diego, CA)], or the two in combination. This concentration of ultra-pure LPS was recommended by the manufacturer based on previous work showing maximum activation of RAW 264.7 cells [37].

Cellular respiration, as an indication of cytotoxicity, was measured by the MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide) assay, which quantifies mitochondrial dehydrogenase activity [38]. Macrophages were plated into 96 well Costar plates at 10⁵ cells per well in 100 μ L of DMEM media. After 4 h of incubation at 37°C for adherence, compounds and DMSO carrier control (0.01% final) were added in triplicate over serial dilutions beginning with 200 μ M per well in a total volume of 200 μ L, and the plates incubated for 24 h. Four h before termination of the assay, each well received 20 μ L of a 5 mg/mL MTT solution in un-supplemented DMEM. After centrifugation, the supernatant for each well was discarded and cells containing reduced MTT were solubilized with 100 μ L of acidified isopropanol (4 mM HCl, 0.1% NP-40 in isopropanol). Following a

brief period of shaking, the optical density (O.D.) for each well was recorded at 550 nm. Each experiment was repeated three times and the data averaged from each triplicate, then expressed as percentage of the control O.D. values for each experiment. For statistical purposes, percentage data were arc sine transformed and compared by ANOVA and Bonferroni's multiple contrast post-hoc tests using Graphpad5. Prior to experiments, an α value of 0.05 was established as statistically significant for cytotoxicity determinants and throughout this study.

2.3 Nitric oxide production as a screening tool for biological activity

Nitric oxide production by LPS-stimulated RAW 264.7 macrophages is used routinely in our lab as a rapid screening assay for anti-inflammatory properties of new pharmacological compounds of interest [35]. Therefore, this assay was first used to demonstrate anti-inflammatory activity of didox, and to determine an appropriate level treatment level in subsequent studies. Raw 264.7 cells in MEM-alpha media lacking phenol red (Gibco) were plated at 10^5 cells/well in 96-well culture plate and grown for 3 h to allow for attachment. Didox was added to the wells in serial dilutions beginning with 200 μ M in the absence or presence of LPS (0.1 μ g/mL). Control cells received 0.01% DMSO. The cells were further incubated for 24 h, after which the supernatants were collected for determination of NO₂ production, a stable non-volatile product of NO production, measured using Griess reagent. Briefly, 100 μ L/well of supernatant were mixed with an equal volume of Griess solution (0.1% naphthylethylenediamine and 1% sulfanilamide in 5% H₃PO₄ solution) at room temperature for 10 min. The absorbance was measured with a micro-ELISA reader at 550 nm. Nitrite concentrations in supernatants were determined by using known amounts sodium nitrite as standard. Subsequent assays used 50 μ M didox, which reduced maximum NO₂ production by approximately 50% (see supplemental data).

2.4 Commercial qRT-PCR arrays for stress and toxicity

Macrophages were seeded at 1.5×10^6 cells/mL in 75 cm² Corning culture flasks and allowed to adhere for 4 h. Cells were then treated for 24 h with 0.01% DMSO carrier as a control, 50 μ M of didox, 0.1 μ g/mL LPS, or both in combination. This experimental regimen was repeated three times and the adhered macrophages scraped from the flask surfaces and pooled (n = 3) per treatment regimen, then harvested by centrifugation to have one pooled pellet from each of the 3 replicated experiments. TRI-reagent® (Molecular Research) was added (1 mL) to cell pellets and the mixture homogenized by gentle pipetting several times. The homogenate was incubated at room temperature for 5 min and 0.2 mL chloroform added. Total RNA was then isolated using procedures outlined by the manufacturer.

After collecting RNA from each tube, genomic DNA contamination was removed using elimination mixture supplied by the manufacturer, and first strand cDNA synthesis was carried out using the RT² Easy First Strand Kit (SABioscience Corporation) as described by the manufacturer. A predesigned Stress & Toxicity Profiler™ array of 96 genes was purchased from SuperArray Bioscience Corporation for use with RT² Real-Timer SYBR Green/fluorescein qPCR master mixes purchased from the same supplier. Specific methods followed those suggested by the manufacturer. RT-qPCR was performed on a BioRad iQ5 real-time PCR detection system. For data analysis, the $\Delta\Delta$ Ct method was used; for each gene

fold-changes were calculated as difference in gene expression between untreated controls and treated cell cultures. Data were gathered and interpreted using software provided by SABioscience. Though the manufacturer suggests that a two-fold change in gene expression to be of significance, a statistical significance was not determined from a single pooled sample – our goal from the pooled sample was to determine which genes may be of interest to follow up with, confirm using regular qRT-PCR, or to guide choices of other endpoints of interest.

2.5 qRT-PCR

The above treatment regimen was repeated, but samples from the three replicated experiments were examined individually and not pooled. RNA was extracted and cDNA obtained from treated murine macrophages as described above. Gene expression, or rather the steady-state level of specific mRNA, was analyzed by quantitative real-time PCR with a BioRad iC5 detection system, RT² SYBR green/fluorescein master mix, and primer sets designed using Integrated DNA Technology (IDT) software, and validated prior to use (Table 1). The quantity of these mRNAs was expressed as fold-changes compared to β -actin expression. For data analysis, the $\Delta\Delta$ Ct method was used. Data were compared between treatment groups using ANOVA, followed by a Bonferroni's post- test using GraphPad5 statistical package.

2.6 Nitric Oxide, IL-6, and IL-10 production

Following a 4 h incubation period for adherence, 10^5 cells/well in 96-well plates were treated for 24 h with 0.01% DMSO as carrier control, 50 μ M didox, 0.1 μ g/mL LPS, or both in a final volume of 200 μ L. Secretion of NO into cell culture supernatants was quantified by two different methods; as described above using the Greiss reagent, and by the fluorescent probe 4,5-diaminofluorescein diacetate (DAF-2 DA) (Molecular Probes, Eugene OR). For the DAF-2 assay, a 10 mM stock solution of the compound in DMSO was diluted in complete media and added at 10^{-5} M to all wells and incubated for an additional 30 min at 37°C to detect total nitric oxide radicals as previously described [39] at ex/Em of 495–515 nm. Mean fluorescence units were determined for the three independent experiments. Interleukin-6 was quantified by mouse READY-SET-GO IL-6 ELISA systems (eBioscience) following the directions provided by the supplier. Interleukin-10 was quantified by mouse IL-10 ELISA MAX systems (Biolegend) following directions provided by the supplier. Data were subjected to an ANOVA and Bonferroni's multiple contrast post-hoc tests using Graphpad5.

2.7 Immunoblotting for iNOS and COX-2 protein expression

Macrophages were seeded in 6-well plates (Costar) at 1.5×10^6 in 3 mL of media containing 0.01% DMSO carrier control, 50 μ M didox, 0.1 μ g/mL LPS, or both compounds in triplicate experiments, and incubated for 24 h. Cells were scraped from the plates and pelleted by centrifugation, then covered with 500 μ L RIPA lysis buffer (50 mM Tris HCl, pH 8, 150 mM NaCl, 1 % NP-40, 0.5% sodium deoxycholate, and 0.1% SDS) containing HaltTM protease inhibitor cocktail (Pierce, Thermo Fisher). The cell pellet was disrupted by gentle vortexing, and incubated on ice for 30 min, then centrifuged at $1000 \times g$ for 10 min. The overlying

supernatant was removed and centrifuged again for 20 min at $14,000 \times g$, and the overlying supernatant removed and its protein content quantified. Twenty five μg of lysate protein from each sample were separated by SDS-PAGE on 10% gels, and transferred overnight onto 0.45 μM nitrocellulose membranes at 4°C . The membranes were washed three times with 0.01 M phosphate buffered saline (PBS, pH 7.2) containing 0.1% tween-20 (PBS-tw20), then covered with blocking buffer (10% FBS in PBS) for 1 h, then washed three times with PBS-tw20. Membranes were then probed for 2 h with goat anti-COX-2 antibody (C-20; Santa Cruz Biotechnology) at 1:750, rabbit anti-mouse iNOS (#610333; BD Transduction Labs) at 1:500, or anti-mouse β -actin (mAb AC-74; Sigma Chemical Company) at 2 μg per ml. Antibodies were diluted in PBS-tw20 containing 1% FCS. The blots were washed three times for 10 min with PBS-tw20, and probed for 2 h with either rabbit anti-goat IgG, goat anti-rabbit IgG, or goat anti-mouse IgG secondary antibody conjugated with alkaline phosphatase (SouthernBiotech) diluted 1:1000 in PBS-tw20 containing 1% FCS. After extensive washings with PBS-tw20, alkaline phosphatase activity was visualized using the substrate NBT-BCIP as means to visualize the relative amount of specific protein. Band densities for each protein were quantified using BioRad G-17 documentation system, and the relative amount of iNOS and COX-2 protein expression were normalized to the β -actin loading control.

2.8 Evaluation of intracellular ROS production to quantify oxidative stress

Cells were seeded at 5×10^5 cells/well in eight chamber cell culture slides (Nalge Nunc Lab-Tek II Chamber System) for 3 h and then treated with didox (50 μM), BSO (400 μM) to deplete glutathione [40], phorbol myristate acetate (PMA) to stimulate oxidative burst activity [41–43], and in combination in the presence or absence of LPS (0.1 $\mu\text{g}/\text{mL}$). After 24 h incubation, dihydroethidium (DHE) (Molecular Probes, Eugene OR) was added at 10^{-5} M to all wells and incubated for an additional 30 min at 37°C to detect total superoxide as previously described [44, 45]. The slides were washed twice with PBS, fixed with chilled absolute methanol for 1 min and mounted using glycerol:PBS (9:1) solution. The cover slip was placed over the mounting medium and the slides were sealed using nail polish. The slides were viewed at ex/em 485–530 nm using Elements Viewer 3.0 software package. Relative fluorescence units (RFU) were subjected to an ANOVA and Bonferroni's multiple contrast post-hoc tests using Graphpad5.

2.9 NF- κ B translocation assays

For immunolabeling of NF- κ B (p65) during nuclear translocation, both cytosolic and nuclear proteins were extracted. Macrophages were treated with 50 μM didox, 0.1 μM LPS, both compounds, or 0.01% DMSO for 3 h. Though it is common to follow NF- κ B translocation as soon as 30 min post-stimulation, maximum translocation is steady for up to 6 hr [46]; a time span that may allow for the translocation of other possible transcription factors, such as AhR ligands [47]. Three hours after LPS-stimulations, cells were removed from flasks by scraping and suspended in 500 μL of buffer A (10 mM HEPES, 10 mM KCl, 1.5 mM MgCl_2 , 1X protease inhibitor solution, 0.05% NP-40, and 0.5 mM DTT, pH 7.9), and incubated on ice for 15 min. The mixture was vigorously vortexed for 10 s and centrifuged at $12,000 \times g$ for 10 min. For nuclear protein extraction, the resulting nuclear pellets were re-suspended in 500 μL of high salt buffer B (5 mM HEPES, 300 mM NaCl, 1.5 mM

MgCl₂, 26% glycerol, 0.2 mM EDTA, 1X protease inhibitor solution, 0.5 mM DTT, pH 7.9) and incubated on ice in a slow shaker for 1 h. The mixture was vigorously vortexed for 15 s followed by centrifugation at 25,000 × g for 20 min. Extract protein concentrations were determined, normalized to a common concentration, and subjected to SDS-PAGE/immunoblotting as described above for iNOS and COX-2 detection. Immunoblots were probed with rabbit anti-p65 antibody (1: 1000) (#RB-1638-PO, Thermo Scientific), and washed x 3 with PBS-tw-20, followed by goat anti-rabbit IgG-AP secondary Ab (1:500) (#T-2191, Invitrogen) incubation for 60 min. The membranes were washed x 3 in PBS-tw-20 and AP activity visualized using BCIP-NBT substrate.

For fluorescence tracking of NF-κβ (p65), macrophages were cultured in 8-chamber slides at 2 × 10⁵ cell/well for 3 h before treatment with 50 μM didox, 0.1 μg/mL LPS, or both, for 3 h. After fixation with 4% PFA, cells were permeabilized with 0.1% Triton X-100/1% BSA in PBS for 30 min. 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 for 1 hr. Cells were incubated overnight with rabbit anti-p65 antibody and washed x 3 with PBS-tw-20, followed by Alexa Fluor® 350 Goat Anti-Rabbit IgG secondary Ab (1:500) (#A-11046, Invitrogen) for 60 min. After 5 min nuclear staining with 0.1 μg/mL DAPI, cells were mounted on slides and analyzed using the Nikon AZ100 Epi-Fluorescent Microscope. Images were captured using the NIS-Elements Viewer 3.0.

3.0 RESULTS

3.1 Cytotoxicity screening

Using mitochondrial dehydrogenase activity as a measure of cytotoxicity [48], the effects of didox on cellular respiration in RAW264.7 were examined over a range of concentrations for 24 h. Cells exposures to 200 μM and below didox, with and without LPS, did not exhibit significant cellular toxicity (data not shown).

3.2 iNOS activity for screening anti-inflammatory activity

Nitric oxide production by LPS-treated cells was a sensitive assay to determine the anti-inflammatory potential of didox for additional studies, with the greatest activity at 50 μM and higher (supplemental data, Figure 1). Therefore, mRNA and protein expression were subsequently evaluated at 50 μM didox.

3.3 Commercial qRT-PCR arrays for stress and toxicity

A commercially available qPCR array platform for evaluating stress and toxicity was used as screening approach to determine if didox modulates LPS-associated changes in mRNA expression in RAW264.7 cells, and to determine possible effects of didox treatment alone. Data from such arrays are expressed as relative fluorescence units, not absolute fold change or mRNA copy number. These data do, however, provide an indication of relative magnitude of expression compared to DMSO controls, and allow a view of relative changes in gene expression associated with inflammation, apoptosis, cell cycle control, and oxidative stress.

Under these experimental conditions, LPS-activated macrophages exhibit an increased expression of several mRNAs associated with activation, signaling, pro-inflammatory, and oxidative stress available on this particular array (Table 2). For each mRNA evaluated on the array, didox alone decreased expression (more than 2-fold suppression), had no effect, or only slightly increased (2-fold or higher), but inhibited expression in combination with LPS. Only for Gpx2 did LPS suppress rather than enhance the expression, yet in the presence of didox the expression of this mRNA was not only restored to normal expression, but enhanced. In almost all cases, didox suppressed the expression of mRNAs enhanced by LPS. Of particular note was the very high expression (> 10-fold) of Ccl2, Csf3, IL1R1, and IL6 in cells treated with LPS, for which expression was sharply suppressed by didox treatment. Several mRNAs were intermediate in expression (> 2-fold, < 10-fold), including Cd40, Ccl3, Csf2, Akt1, C3, Sod2, Edg2, Egr1, TLR3, TLR4, IRAk1, IRAK2, MAPK3, MAPK1, Tnfsf10, and Lta. Of note from the array, expression of Myd88, which is typically associated with LPS-activated macrophages, was not changed by LPS treatment under these experimental conditions.

3.4 qRT-PCR

qRT-PCR was carried out to validate the effects of treatments on IL-6 mRNA expression, as a keystone cytokine associated with inflammation and systemic acute phase responses, and was highly expressed in the above commercial array. Also quantified, were expression profiles of COX-2, iNOS, and TNF- α , as well as p65 and p38. In addition, SOD1 and CAT, two products associated with oxidative stress were evaluated. As expected, LPS was a potent inducer for expression of IL-6, iNOS, TNF- α , and COX-2 (Figure 2). Didox inhibited LPS-induced IL-6, COX-2, and iNOS expression, with the most impact on iNOS and TNF- α . Expression of both p65 and p38 were enhanced by LPS treatment, and reduced by treatment with didox (Figure 3). Expression of p65 mRNA was nearly 10-fold higher than p38 in LPS-treated cells. The effects of didox and LPS on the expression of SOD1 and CAT mRNA were different in that didox alone was a potent stimulator for CAT expression, but only modestly so for SOD1 expression, while LPS alone was more potent in inducing SOD1 than CAT (Figure 5). However, didox enhanced the expression of both genes in combination with LPS, thereby possibly providing more anti-oxidant properties to cells.

3.5 Nitric oxide production, IL-6 and IL-10 secretion

LPS-stimulated macrophages are a key source of iNOS enzymatic activity, which is easily, quantified using both standard colorimetric and fluorometric techniques. Using the Greiss colorimetric reagent system, and under the conditions of experiments, LPS was a potent stimulator for production of NO by iNOS (Figure 5A, B). In the presence of didox, NO production was reduced by approximately 5-fold. Using the fluorescent dye DCF-2 DA as an indicator of cellular production of NO $\cdot$ radical, it is evident that LPS is stimulatory, and that didox treatment suppressed production by approximately 3-fold. Also observed, LPS is a potent inducer of IL-6 and IL-10 secretion in macrophages (Figure 5C, D), with the production of IL-6 being higher than IL-10. Didox treatment resulted in little effect on basal IL-6 and IL-10 secretion, but suppressed secretion stimulated by LPS. IL-6 secretion was more dramatically affected by didox than was secretion of IL-10.

3.6 Immunoblotting for iNOS and COX-2 protein expression

LPS was a strong inducer of both iNOS and COX-2 at the protein level at 24 h of treatment (Figure 6). Didox did not induce expression of either protein, but reduced LPS-induced expression of both. When band densities were compared for all three replicate experiments, iNOS expression was more affected by didox treatment than was COX-2.

3.7 Evaluation of ROS production under conditions of PMA-stimulation and glutathione depletion

Total reactive oxygen species (ROS) production was determined using a fluorescence assay after treatment with 50 μ M didox in the presence or absence of 400 μ M BSO as a glutathione depleting agent, and in the presence or absence of 0.1 μ g/mL LPS or 0.1 μ M PMA to stimulate the oxidative burst response. Both LPS and PMA stimulated the production of ROS, and BSO treatment elevated basal ROS production (Figure 7). The highest level of ROS production was in cells treated simultaneously with PMA and BSO. In each case, didox was able to reduce ROS production to near baseline levels, suggesting either an ability to inhibit NADPH-oxidase activities that lead to ROS production, or by inhibiting the depletion of glutathione.

3.8 NF- κ B (p65) activation and translocation assays

To better define possible mechanisms by which didox may affect oxidative stress and pro-inflammatory profiles, macrophages were incubated with 50 μ M didox, 0.1 μ M LPS, or both for 24 h and NF- κ B (p65) protein expression examined in both the cytosol and nucleus. After a 3 h incubation period, more fluorescence and protein were observed in the cytosol than in nuclei of untreated and treated cells, whereas the reverse was observed in LPS-treated cells (Figure 8). Didox also reduced p65 presence in nuclei since less fluorescence and protein were observed in nuclei of LPS-stimulated cells, while more fluorescence occurred in the cytosol. The presence of more p65 in cytosol of LPS-treated cells suggests that didox limited translocation of LPS-induced NF- κ B nuclear translocation. Likewise, the same over-all trend can be seen in immunoblots of p65 protein from cytosolic vs. nuclear fractions.

4.0 DISCUSSION

In this study we examined the effects of didox on pro-inflammatory and oxidative stress profiles in the murine macrophage cell line RAW264.7 as a model for predicting the benefits of therapy for this compound in multiple inflammatory disease states. Didox was especially potent at suppressing iNOS expression and activity, cytokine mRNA and protein expression, and reducing ROS. Under the conditions of our study, didox was not cytotoxic to macrophages when given alone over a broad range of concentrations as high as 200 μ M, nor when given in combination with 0.1 μ g/mL LPS. As shown in Table 1, LPS treatment greatly enhanced the expression of genes representative of the classical view of a localized inflammatory response following both tissue damage and entry of pathogens, thereby activating macrophages through TLRs, DAMP receptors, and other receptors recognizing PAMPs [49, 50]. In this scenario, histiocytes would secrete chemokines, such as CCL2 and CCL3 to recruit monocytes and neutrophils, CSF-2 and CSF-3 for the promotion of rapid granulocyte and macrophage hematopoiesis, and complement components such as C3,

which is critical to classical-, alternative-, and lectin-associated complement fixation. LPS typically activates through TLR-4, with additional activation of type 1 interferon signaling, and subsequent activation of signaling cascades involving several kinases and NF- κ B, with expression and secretion of pro-inflammatory cytokines and expression of receptors for these cytokines [51, 52]. Also involved in LPS signaling are the mitogen-activated protein kinases, including p38 α , or MAPK14 [53] that lead to inflammatory cytokine production. Of particular note, Didox reduced expression of genes typically associated with local inflammatory responses without cytotoxicity. Overall, these screening qRT-PCR array data strongly suggests that didox has great potential to modulate typical inflammatory profiles associated with macrophage functions in general.

Validation of the PCR array by qPCR of iNOS, IL-6, TNF- α , and COX-2 mRNA levels at the time of sampling clearly demonstrates a potent inhibition of LPS-stimulated expression by didox, and even suppression of mRNA levels by didox in the absence of LPS. While our observations offer no single mode of action associated treatment, one can speculate that inhibition of NF- κ B activation and signaling is involved, which is supported by the observed suppression of LPS-stimulated p65 in qPCR assays. It has been suggested that P13K activation following LPS binding to TLR-4 is also involved in activation of p38 α and NF- κ B [52]. The inhibition of expression of both p65 and p38 α by didox alone further supports the observed suppression of IL-6, iNOS, and COX-2 mRNA and protein products, and TNF- α mRNA; four classical gene products associated with inflammation. Moreover, we show by both western blotting and immunofluorescence that NF- κ B (p65) was retained in the cytosol of cells treated with didox. Though not examined in this study, we predict that p38 α activation and translocation would be similarly inhibited by didox.

The search for therapeutic drugs and experimental compounds targeting NF- κ B signaling, either by inhibition of IKK β degradation or impaired transcription, is intense [54], and especially since there is more than one pathway associated with NF- κ B signaling [55]. The LPS-macrophage model used in our study herein is considered the canonical pathway, involving the degradation of three IkK units into p50/Ik β - α /RelA units that result in p50/RelA being released as the functional NF- κ B unit. This resulting p50 and RelA (p65) partnership as a transcription factor is critical to innate immunity, inflammation, and cell survival [6, 7, 54], and in macrophages involves the release of pro-inflammatory cytokines in response to ligand binding to most TLRs. The observation that didox diminished all indices of LPS-induced TLR-4 activation, including transactivation of p65 (RelA) from the cytosol to the nucleus, strongly suggests that NF- κ B is a primary target. The alternative NF- κ B way involves p52/RelB as partners for transcription, but is not activated by TNF- α , IL-1, and LPS, three key activators in the canonical pathway [54]. To date, we have not examined the effects of didox on the alternative pathway, which is critical to aspects of lymphoid development, B-cell maturation, and humoral immunity [54], but our data strongly support earlier suggestions that NF- κ B signaling is a key target of didox [30]

Chronic infection and inflammation, excessive ROS production by phagocytes, hypoxia/re-perfusion cycles, DNA damage, drug abuse, and other health maladies are associated with oxidative stress induced NF- κ B (p65) activation [56–59]. As demonstrated in the gene array data, expression of superoxide dismutase was enhanced by LPS treatment, and even more so

with the combination of LPS and didox. LPS treatment appears to severally stress the overall redox state of the cell because glutathione peroxidase expression is suppressed, yet didox reverses this phenomenon. Through qPCR, we show that didox is a potent stimulator of catalase gene expression, and counters the effects of LPS on the expression of this critical anti-oxidant. Like-wise, superoxide dismutase (SOD1) is increased by LPS, but the effects of didox on expression is dramatically higher than what is seen for catalase, and as with catalase, didox increases expression above what is induced by LPS.

The effect of didox on redox gene expression is complemented by reactive oxygen species (ROS) assays in which LPS- and PMA-induced superoxide radical production was monitored in the presence of didox and BSO, a potent compound known to deplete the cell of glutathione, a key player in maintaining a proper redox state in cells. Just how didox could both inhibit LPS-activation of macrophages through NF- κ B, and probably p38 α as well, while also inducing antioxidant enzymes and reducing ROS remains unclear. As noted in our study herein, didox reduces ROS production during macrophage activation by LPS, and by doing so possibly ameliorates the ability of LPS to activate NF- κ B through oxidative stress generated during the activation process. This may explain the effectiveness of didox in a myriad of models of inflammation and models of both infectious and non-infectious diseases previously cited. Though not explored in this study, there is the possibility that didox alters the NADPH oxidase complex of RAW 264.7 cells, and perhaps through inhibiting the association of cytosolic components (p47phox, p67phox, and p40phox) with membrane components (gp91phox and p22phox), a tightly controlled system for producing superoxide anion upon activation [59]. To this point, superoxide anion is the primary oxygen radical recognized by our DHE assay [44, 45]. Future studies should focus on the NADPH oxidase system by examining the effects of didox on expression of both mRNA and proteins, and assembly thereof from cytosol to the membrane.

At the core of the spectrum of physiological functions of macrophages in both normal and disease states is the ability of these cells to become polarized towards two broad functional categories, M1 vs M2 cells [60–62]. Such plasticity and tendency for M1 vs. M2 polarization is analogous to Th1 vs. Th2 T-cell responses, respectively [63], and the interaction between subtypes is probably very similar in terms of one phenotype regulating immune responses of the other. Polarization of macrophages by didox was not directly examined in this study, but suppression of typical M1 biomarkers, such as high iNOS activity and high expression of iNOS, TNF- α , IL-6, and COX-2 genes without direct cytotoxicity indicates promise for this compound in treating a variety of inflammatory diseases. This attribute is not unlike the role for aspirin in ameliorating symptoms of chronic inflammation, both locally and systemically. The benefit of didox over aspirin is that the former is well tolerated by patients without gastro-intestinal issues associated with the latter.

Taken together, the experiments outlined in this study reveal a potent effect of didox on inflammation profiles and ROS production in RAW264.7 macrophages, with iNOS activity and ROS generation being especially sensitive to treatment in vitro. Didox is well tolerated both in vitro and in vivo, and in clinical trials, and as such should be considered as a front-line drug for ameliorating inflammation in humans, though more studies are needed under clinical observation. Moreover, didox may have potential as adjuvant therapy in situations

where treatments with primary drugs may harbor inflammatory properties on their own, as exemplified by valproic acid treatments for epilepsy [64] and halothane hepatitis [65].

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Highlights

- Didox is a potent inhibitor of IL-6 secretion in RAW264.7 macrophages
- Didox suppresses the expression of COX-2, iNOS, IL-6, and TNF- α gene expression
- Didox ameliorates or scavenges reactive oxygen species production by LPS treatment
- Didox enhances the expression of SOD1 and catalase genes

3,4-Dihydroxy-Benzohydroxamic Acid (Didox)

Figure 1.

Molecular structure of 3,4-Dihydroxy-Benzohydroxamic Acid (didox)

Figure 2.

Didox inhibits the expression of LPS-induced iNOS, IL-6, TNF- α , and COX-2 mRNA expression in RAW264.7 macrophages. Cells were treated with 50 μ M didox, 0.1 μ g/mL LPS, or both in combination, for 24 h. Data represent the mean fold expression $\pm$ S.E. of three different experiments. (*) indicates $p < 0.05$.

Figure 3.

Didox inhibits the expression of LPS-induced p65 and p38α genes in RAW264.7 macrophages. Cells were treated with 50 μM didox, 0.1 μg/mL LPS, or both in combination, for 24 h. Data represent the mean fold expression ± S.E. of three different experiments. (*) indicates $p < 0.05$.

Figure 4.

Didox modulates the expression of catalase and SOD1 genes in RAW264.7 macrophages. Cells were treated with 50 μ M didox, 0.1 μ g/mL LPS, or both in combination, for 24 h. Data represent the mean fold expression $\pm$ S.E. of three different experiments. (*) indicates $p < 0.05$.

Figure 5.

NO, IL-6, and IL-10 secretion by RAW264.7 macrophages following 24 h treatment with 50 μM didox, 0.1 μg/mL LPS, or both in combination. Nitric oxide production was inhibited by didox as measured by total accumulated nitrite accumulation using the Greiss reagent assay (A), and by fluorescence (relative fluorescence units) using the probe DCF2-CA (B). Didox also inhibited IL-6 (C) and IL-10 (D). Each value represents the mean ± S.E. of three experiments performed in triplicates. (*) indicates $p < 0.05$.

Figure 6. Representative western blotting of COX-2 and iNOS expression in RAW264.7 macrophages following treatment with 50 μ M didox, 0.1 μ g/mL LPS, or both in combination for 24 h (A). Graphical representation of COX-2 and iNOS expression from three independent experiments (B). Data represent the mean relative fold expression $\pm$ S.E. of three different experiments. (*) indicates $p < 0.05$

Figure 7.

Didox inhibits ROS generation in Raw 264.7 murine macrophages. Cells were treated with 50 μ M didox in the presence or absence of LPS (0.1 μ g/mL), BSO (200 μ M), PMA, or in combinations. After 24h incubation, 10 μ M DHE was added to all wells and incubated for an additional 30 min at 37°C. Mean fluorescence intensities were measure in various fields in triplicates. Data represent the mean relative fluorescence intensity $\pm$ S.E. of three different experiments. * indicates $p < 0.05$.

Figure 8. Didox attenuates LPS-induced nuclear translocation of p65 in RAW264.7 macrophages. Cells were treated with 50 μ M didox, 0.1 μ g/mL LPS, or both in combination, for 3 h, followed by immunoblotting for cytosolic (A) and nuclear p65 protein (A). Intracellular distribution of p65 was quantified by immunofluorescence (B). Data represent the mean fluorescence intensity staining $\pm$ S.E. of three different experiments. (*) indicates $p < 0.05$.

Table 1

Results from commercial qRT-PCR Stress & Toxicity™ arrays targeting select genes associated with inflammation and oxidative stress. RAW 264.7 cells were treated with 50 µM Didox, 1 µg/ml LPS, Didox + LPS, or 0.01% DMSO as the carrier control for 24 h. Cells were harvested and processed for analysis of gene expression using designed commercial qRT-PCR arrays (SuperArray®) following instructions provided by the manufacturer. Data for gene expression represent x-fold changes in mean SYBER-green fluorescence units of 3 pooled samples compared to carrier control-treated cells, and normalized for house-keeping gene expression. Details are provided in the Methods and Materials section.

Gene Name	GenBank #	Symbol	DX	LPS	LPS+DX
CD40 on APC	NM_011611	Cd40	-2.81	7.37	3.76
Chemokine ligand 2	NM_011333	Ccl2	-4.12	23.40	-1.54
Chemokine ligand 3	NM_011337	Ccl3	-1.57	6.93	1.65
Colony stimulating factor 2	NM_009969	Csf2	-3.42	6.30	1.81
Colony stimulation factor 3	NM_009971	Csf3	-13.47	92.33	3.14
Complement component 3	NM_009778	C3	-1.67	2.82	-1.43
Chemokine ligand 10	NM_021274	Cxcl10	-5.98	3.23	-4.86
Interleukin-1 receptor type 1	NM_008362	IL1R1	-1.74	10.35	2.70
Interleukin 6	NM_031168	IL6	-9.26	26.49	7.21
Toll-like receptor 3	NM_126166	TLR3	-1.11	4.98	3.40
Toll-like receptor 4	NM_021297	TLR4	-2.84	2.75	-2.07
IL-1 receptor-associated kinase 1	NM_008363	IRAK1	-1.61	9.71	-2.11
IL-1 receptor-associated kinase 2	NM_172161	IRAK2	-1.88	3.08	-1.63
MAP Kinase Kinase Kinase	NM_011945	MAP3K1	-1.36	2.71	-1.78
MAP Kinase 3	NM_011952	MAPK3	1.10	5.15	1.12
Myeloid differentiation factor 88	NM_010851	Myd88	-1.63	1.59	1.05
TNF-related apoptosis-inducing ligand	NM_009425	Tnfsf10	-1.81	7.72	1.24
Lymphotoxin-alpha	NM_010735	Lta	-1.21	3.56	2.73
Thymoma viral proto-oncogene 1	NM_009652	Akt1	-8.35	4.14	-2.91
Lysophosphatidic acid receptor	NM_010336	Edg2	2.17	3.02	6.30
Early growth response 1	NM_007913	Egr1	-1.07	4.87	-4.41
Superoxide dismutase 2	NM_013671	Sod2	2.00	7.09	13.64
Glutathione peroxidase 2	NM_030677	Gpx2	2.25	-3.09	5.23

Table 2

Primer sets for qRT-PCR.

Gene Name	Accession #	Primer Sequence (5'→3')	Tm°C	Product size (bp)
GAPDH	NM_008084	F: TGT GAT GGG TGT GAA CCA CGA GAA R: ACC CTG TTG CTG TAG CCG TAT TCA	57	115
IL-6	NC_000071.5	F: TTG TAC AGT CCC AGT CAG GCA ACA R: TCA AGC TAC TGC AGG CCA GTT ACA	58	80
COX-2	NC_000067.5	F: GCC AGC AAA GCC TAG AGC AAC AAA R: TAC TGA GTA CCA GGC CAG CAC AAA	57	144
IL1-β	NM_008361	F: TGG AGA GTG TGG ATC CCA AGC AAT R: TGC TTG TGA GGT GGT GAT GTA CCA	58	137
TNF-α	NM_013693	F: TGA GTT CTG CAA AGG GAG AGT GGT R: TGC ACC TCA GGG AAG AAT CTG GAA	57	110
iNOS	NM_010927	F: CAA ACA CGA GTG CAG CTG GTT GAA R: AGG CAG GAC TGA GTT CAG TGT GTT	58	115
SOD1	NM_011434	F: CGGATGAAGAGAGGCAATGTT R: CACCTTTGCCCAAGTCATCT	56	100
p38a	NM_011951	F: TGC GTC TGC TGA AGC ACA TGA AAC R: ATGGGTCACCAAGGTACACGTCATT	57	107
p65	NM_009045.4	F: TGT GGA GAT CAT CGA ACA GCC GAA R: TGT TCC TGG TCC TGT GTA GCC ATT	57	154
Catalase9	NM_009804.2	F: AAG ACA ATG TCA CTC AGG TGC GGA R: GGC AAT GTT CTC ACA CAG GCG TTT	57	83