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3,4-Dihydroxy-benzohydroxamic acid (Didox) suppresses pro-inflammatory profiles and oxidative stress in TLR4-activated RAW264.7 murine macrophages

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ABSTRACT

Didox (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 didox may have anti-oxidative stress-like properties, while other studies hint that didox 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 didox is very potent at levels as low as 6.25 μM, with maximal inhibition at 100 μM. A qRT-PCR array was then employed to screen didox for other potential anti-inflammatory and anti-oxidative stress-related properties. Didox was very potent in suppressing the expression of these arrayed mRNA in response to LPS, and in some cases didox alone suppressed expression. Using qRT-PCR as a follow up to the array, we demonstrated that didox suppresses LPS-induced mRNA levels of iNOS, IL-6, IL-1, TNF-α, NF-κβ (p65), and p38-α, after 24 h of treatment. Treatment with didox 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 didox. Moreover, we demonstrate that nuclear translocation of NF-κβ (p65) in response to LPS is inhibited by didox. These findings were supported by qRT-PCR for oxidative stress genes SOD1 and catalase. Overall, this study supports the conclusion that didox may have a future role in managing acute and chronic inflammatory diseases and oxidative stress due to high production of ROS.

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-κβ, 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)

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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 diorders [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-κβ 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-κβ signaling.

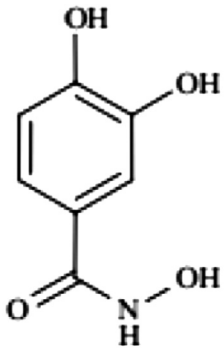
2. Materials and methods

2.1. Cells and cell culturing

The murine macrophage cell line RAW264.7 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 μg/mL streptomycin, 110 μg/mL sodium pyruvate, 1% non-essential amino acids (100 X stock), 4.5 g/L glucose, and 1.5 g/L of $NaCO_3$, each from Sigma (Sigma Aldrich, St. Louis MO, USA). Cells were typically grown and maintained at 37 °C with 5% CO_2 in Corning 75 cm^2 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 Fig. 1. Unless otherwise noted, cells were treated with Didox alone, with 0.1 μg/mL LPS [*Escherichia 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 RAW264.7 cells [37].



3,4-Dihydroxy-Benzohydroxamic Acid (Didox)

Fig. 1. Molecular structure of 3,4-dihydroxy-benzohydroxamic acid (didox).

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 10^5 cells per well in 100 μL 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 μM per well in a total volume of 200 μL, and the plates incubated for 24 h. Four h before termination of the assay, each well received 20 μL 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 μL 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 RAW264.7 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. Raw264.7 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 μM in the absence or presence of LPS (0.1 μg/mL). Control cells received 0.01% DMSO. The cells were further incubated for 24 h, after which the supernatants were collected for determination of NO_2 production, a stable non-volatile product of NO procution, measured using Griess reagent. Briefly, 100 μl/well of supernatant were mixed with an equal volume of Griess solution (0.1% naphthylethylenediamine and 1% sulfanilamide in 5% H_3PO_4 solution) at room temperature for 10 min. The absorbance was