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Didox (3,4-dihydroxybenzohydroxamic acid) suppresses IgE-mediated mast cell activation through attenuation of NF κ B and AP-1 transcription

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Abstract

Mast cell activation via the high-affinity IgE receptor (Fc ϵ RI) elicits production of inflammatory mediators central to allergic disease. As a synthetic antioxidant and a potent ribonucleotide reductase (RNR) inhibitor, Didox (3,4-dihydroxybenzohydroxamic acid) has been tested in clinical trials for cancer and is an attractive therapeutic for inflammatory disease. We found that Didox treatment of mouse bone marrow-derived mast cells (BMMC) reduced IgE-stimulated degranulation and cytokine production, including IL-6, IL-13, TNF and MIP-1 α (CCL3). These effects were consistent using BMMC of different genetic backgrounds and peritoneal mast cells. While the RNR inhibitor hydroxyurea had little or no effect on IgE-mediated function, high concentrations of the antioxidant N-acetylcysteine mimicked Didox-mediated suppression. Furthermore, Didox increased expression of the antioxidant genes superoxide dismutase and catalase, and suppressed DCFH-DA fluorescence, indicating reduced reactive oxygen species production. Didox effects were not due to changes in Fc ϵ RI expression or cell viability, suggesting it inhibits signaling required for inflammatory cytokine production. In support of this, we found that Didox reduced Fc ϵ RI-mediated AP-1 and NF κ B transcriptional activity. Finally, Didox suppressed mast cell-dependent, IgE-mediated passive systemic anaphylaxis *in vivo*. These data demonstrate the potential use for Didox as a means of antagonizing mast cell responses in allergic disease.

Keywords

allergic inflammation; mast cells; IgE; Fc ϵ RI; Didox; NF κ B; AP-1

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1. Introduction

Mast cells are most recognized for their role in allergic disease. Antigen-induced cross-linkage of surface-bound IgE activates the high-affinity IgE receptor, FcεRI, and leads to the two distinct phases of mast cell activation [1–6]. The first phase, degranulation, occurs immediately and is noted by release of preformed inflammatory mediators such as histamine and proteases. The second phase occurs several hours later, and is marked by *de novo* production and secretion of arachidonic acid metabolites and many cytokines, including IL-4, IL-6, IL-13, MIP-1a, MCP-1, TNF, GM-CSF and VEGF. While these mediators are necessary for inflammation associated with acute allergic responses, recent evidence suggests that chronic mast cell activation also contributes to harmful inflammatory diseases such as rheumatoid arthritis, multiple sclerosis and heart disease [7–10]. As such, mast cells have become an important target for therapeutic intervention in these maladies.

The synthetic antioxidant and potent ribonucleotide reductase (RNR) inhibitor Didox (3,4-dihydroxybenzohydroxamic acid) has become an attractive therapeutic for treatment of inflammatory diseases [11–13]. Originally developed as an antineoplastic and antiproliferative agent to improve upon the activities of hydroxyurea, Didox possesses both iron chelating and free-radical scavenging function. Didox exhibits greater RNR inhibition than hydroxyurea, with minimal *in vivo* toxicity [11,14–16]. In addition to its anti-neoplastic activity, more recent studies have shown suppressive effects on immune cell activation. Inayat and colleagues found that Didox suppresses T cell proliferation and cytokine production following anti-CD3 activation that models organ rejection or graft-versus-host disease [17]. Didox treatment of LPS-stimulated RAW264.7 macrophage cells *in vitro* reduced the expression of inflammatory genes without causing cytotoxicity [18]. Furthermore, we recently published that Didox suppresses IL-33-mediated mast cell activation *in vitro* [19]. These observations prompted us to study Didox effects on IgE-mediated mast cell activation. Here we report that Didox antagonizes IgE-induced degranulation, cytokine production, transcription factor function, and passive systemic anaphylaxis. These data support further study of this drug's potential for understanding and treating allergic disease.

2. Material and Methods

2.1. Reagents

3,4-Dihydroxybenzohydroxamic acid (Didox) was synthesized by Molecules for Health, Inc. (Richmond, VA). Lyophilized Didox was resuspended in DEPC-treated water at concentrations of 100mM, briefly sonicated, and filter sterilized (0.45mm syringe filter, Cell Treat). Didox was added to cultures at a final concentration of 100μM unless otherwise indicated. Recombinant mouse IL-3 and SCF were purchased from Biolegend (San Diego, CA). DNP-specific purified mouse IgE was purchased from BD Pharmingen (San Jose, CA). Dinitrophenyl-coupled human serum albumin (DNP-HSA), propidium iodide, N-acetylcysteine (NAC), and hydroxyurea (HU) were purchased from (Sigma, St Louis, MO).

2.2. Mice

Most experiments utilized C57BL/6J mice purchased from The Jackson Laboratory (Bar Harbor, ME) and used at a minimum of 10 weeks old with approval from the Virginia Commonwealth University Institutional Animal Care and Use Committee. To ensure the reproducibility of findings in another genetic background, 129/SvJ mice were also used in Figure 3.

2.3. Mast cell culture

Most experiments were conducted using mouse bone marrow-derived mast cells (BMMC), which were derived by culture for at least 21 days in complete RPMI supplemented as previously described, with 90% FceRI⁺ and cKit⁺ mast cells present by day 21 [19–21]. Cultures were used for up to 8 weeks of age and age-matched in experiments. Additionally, mast cells from mouse peritoneal lavage were expanded in complete RPMI 1640 medium containing 10% FBS and IL-3+SCF (10 ng/mL) for 7–10 days to yield ~85% FceRI⁺ and cKit⁺ mast cells. These cells matured *in vivo* with *ex vivo* expansion and were used to support BMMC data to ensure results were not an effect of *in vitro* differentiation.

2.4. IgE-mediated activation

Mast cells were sensitized overnight with 0.5mg/mL anti-DNP mouse IgE (k isotype). Next, cells were washed and resuspended at 1×10⁶ cells/mL with IL-3 and SCF (10ng/mL). Crosslinking (XL) was induced by the addition of DNP-HSA (50ng/mL) for 18 hours. Didox or vehicle control (DEPC water) was added for 6 hours prior to IgE activation unless otherwise stated.

2.5. ELISA

Following Didox treatment and IgE activation for 18 hours, cytokine levels were measured in the cell culture supernatant via ELISA. Murine ELISA kits were purchased from BioLegend (San Diego, CA) for IL-6, TNF, and MCP-1 (CCL2) and Peprotech (Rocky Hill, NJ) for IL-13 and MIP-1a (CCL3). ELISAs were performed using duplicate samples according to the manufacturers' protocols.

2.6. mRNA analysis

BMMC were activated by IgE XL for 4 hours for IL-6 analysis or 24 hours for SOD1 and catalase analysis. Cells were harvested and total RNA was extracted with TRIzol reagent (Life Technologies, Grand Island, NY). Nucleic acid was quantified and assessed for purity using the Thermo Scientific NanoDropTM 1000 UV-vis Spectrophotometer (ThermoScientific, Waltham, MA). For mRNA detection, cDNA was synthesized using qScriptTM cDNA Synthesis from Quanta Biosciences (Gaithersburg, MD). BioRad CFX96 Touch Real-Time PCR Detection System (Hercules, VA) was used to amplify message using PerfeCTa SYBR Green SuperMix (Quantabio, Gaithersburg, MD). The following primers were purchased from Eurofins MWG Operon (Huntsville, AL): IL-6 (forward: 5'-TCCAGTTGCCTTCTTGGGAC-3'; reverse, 5'-TCCAGTTGCCTTCTTGGGAC-3'), GAPDH (forward: 5'-GATGACATCAAGAAGGTGGTG-3', reverse: 5'-GCTGTAGCCAAATTCGTTGTC-3'), SOD1 (forward: 5'-

CGGATGAAGAGAGGCATGTT-3', reverse: 5'-CACCTTTGCCCAAGTCATCT-3'), catalase (forward: 5'AAGACAATGTCACTCAGGTGCGGA3', reverse: 5'-GGCAATGTTCTCACAGAGGCGTTT-3'), and β -actin (forward: 5'-GATGACGATATCGCTGCGC-3'), reverse: 5'-CTCGTCACCCACATAGGAGTC-3'). Amplification conditions consisted of a heat-activation step at 95°C for 2 minutes followed by 40 cycles of 95°C for 15 seconds, 55°C for 30 seconds and 60°C for 1 minute. All melting curve analysis was performed between 50°C and 95°C. Results were normalized to the housekeeping gene GAPDH or actin and the DEPC treated water control using the Livak Method.

2.7. Flow cytometry

For all experiments, flow cytometry was run on the FACSCelesta (BD Biosciences). For cell cycle analysis, propidium iodide DNA staining was used. Cells were fixed at 4°C for at least 4 hours in the dark in Fixation Buffer (52.5% ethanol, 12.5% fetal calf serum, 35% 1X PBS). Following fixation, cells were washed and stained in a solution of PBS, RNase A (100mg/ml), EDTA, and propidium iodide (200mg/mL) for 2–3 hours in the dark before flow cytometric analysis. For assessing viability, propidium iodide was added to cells at a final concentration of 10 mg/mL immediately before flow cytometric analysis. For degranulation, APC-anti-CD107a (2 mg/ml, clone 1D4B, Biolegend), PE-anti- mouse CD63 (2 mg/mL, clone NVG-2, Biolegend), their respective isotype controls (APC-anti-IgG2a (clone RTK2758, Biolegend) and PE rat IgG2b (BD Pharmagen)), and anti-CD16/32 (10 mg/ml, clone 2.4G2, BD Pharmingen) were added to the cell culture media ~30 min prior to IgE activation. Following 10 minute activation, cells were washed and analyzed by flow cytometry. For analysis of oxidative stress, cells were treated with Didox for 6 hours, then resuspended in Hank's buffered saline solution (HBSS) + 2',7' Diochlorofluorescein Diacetate (DCFH-DA, 5 μ M, Millipore) $\pm$ Didox for 30 minutes at 37°C. Cells were then activated with DNP (50 ng/mL) for 2 hours, washed, and analyzed in the FITC channel by flow cytometry. For surface expression of Fc ϵ RI, anti-CD16/32 (clone 2.4G2) and APC-anti-Fc ϵ RI (2 mg/ml, clone MAR1, Biolegend) were added to pelleted cells, incubated for 30 minutes at 4°C, washed in FACS buffer (PBS, 3% FBS, 0.1% sodium azide), and analyzed by flow cytometry.

2.8. Luciferase reporter assay

BMMC (3×10^6) were co-transfected with pGL4.74 [hR/luc/TK] encoding the luciferase gene from *Renilla reniformis* under the HSV-TK promoter, and either pGL4.32[luc2p/NF κ B RE/Hygro] or pGL4.44[luc2p/AP1 RE/Hygro] vectors encoding luciferase gene from *Photinus pyralis* (Firefly) under AP-1 and NF κ B response elements, respectively. The co-transfection protocol utilized a 1:5 ratio of hR/luc:NF κ B/AP1. The protocol was as previously described [22] using the Amaxa Nucleofector (Lonza, Allendale, NJ). All experiments were performed 48 hours after transfection. Luciferase expression within cell lysates was measured with the Dual-Luciferase Reporter Assay System and the GloMax 20/20 Luminometer (Promega, Madison, WI).

2.9. Passive systemic anaphylaxis

Age-matched groups of mice (~9 months old) were sensitized overnight with anti-DNP IgE (50 µg) intraperitoneal (IP) injection. The next day, these mice were IP injected with PBS or Didox (350 mg/kg) 6 hours prior to an IP injection of PBS or DNP-HSA (100 mg) to elicit anaphylaxis. The core body temperature of each mouse was measured using a rectal microprobe for 2 hours (Physitemp Instruments).

2.10 Statistical analyses

For Figures 1–6, a one-way analysis of variance (ANOVA) was used to detect differences between groups. For Figure 7, a two-way ANOVA was used to detect differences between groups over time. Post hoc testing with Tukey's multiple comparisons was used to determine which conditions were significantly different from the vehicle control. GraphPad Prism software was used for statistical analyses. Data are expressed as mean and standard error of mean (SEM), with statistical significance set at * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$. NSD denotes no significant difference.

3. Results

3.1. Didox suppresses IgE-mediated BMMC activation

Didox has previously been shown to suppress cytokine production by anti-CD3-activated T-cells or LPS-activated RAW264.7 macrophages [17,18]. To determine Didox's effect on IgE-mediated mast cell activation and the optimal treatment conditions, time-course and dose-response experiments were performed. To determine time-dependent effects, IgE-primed BMMC were pretreated $\pm$ Didox (100 µM) for 24-, 6-, or 0-hr, then activated via IgE cross-linking (XL). Didox suppressed IL-6 and IL-13 production, with optimal effects occurring when Didox was added 6 hours prior to antigen (Figure 1A). To determine dose-dependent effects, IgE-primed BMMC were treated $\pm$ Didox at multiple doses for 6 hrs and activated via IgE cross-linking (XL). Significant suppression was achieved at concentrations ranging from 50–200µM (Figure 1B). The treatment parameters of 100µM Didox for 6-hr and 24-hr (total length of experiment with activation) were assessed for effects on cell cycle, cell viability, and FcεRI receptor expression. While BMMC are slow to divide (~once every 4 days), Didox is an antiproliferative agent and we wanted to ensure that differences observed in treatment groups were not due to changes in cell cycle. Only very small (2%) changes were observed following 24-hr Didox treatment (Supplementary Fig 1A), which are unlikely to account for a 50% reduction in cytokine production. Additionally, there was no effect on cell viability or FcεRI expression by surface staining after 24-hr Didox treatment (Supplementary Figure 1B and C), suggesting that reduced cytokine production is not simply due to cell death or receptor loss. Therefore, all further experiments were completed using 100µM Didox treatment for 6 hours.

In addition to IL-6 and IL-13 (Figure 1A and 1B), Didox suppressed TNF and MIP-1a (CCL3) secretion, but had no significant effect on MCP-1 (CCL2) (Figure 1C). To address if Didox suppresses cytokine production at the level of gene transcription or translation, IL-6 mRNA was analyzed by RT-qPCR. Didox significantly antagonized IL-6 mRNA induction relative to GAPDH, suggesting suppression is at the level of cytokine gene induction (Figure

1D). These data indicate Didox suppresses newly-synthesized cytokines and chemokines that comprise the late phase of the mast cell response. To measure rapid degranulation, we assessed changes in surface expression of CD107a and CD63, markers which we and others have used previously [23–26]. We typically see that upon cross linking, the percent of CD107 positive cells increases as well as the mean fluorescence intensity (MFI) of CD63, as CD63 is constitutively expressed on BMMC while CD107a is not. IgE-primed BMMC were treated $\pm 100\mu\text{M}$ Didox for 6 hours, followed by antigen-mediated crosslinking for 10 minutes. Increases in percent CD107a+ and CD63 MFI were significantly suppressed among Didox-treated versus vehicle-treated BMMC (Figure 1E). Collectively these results show Didox inhibits both the early and late phases of mast cell activation.

3.2. Didox suppresses FcεRI-mediated cytokine production by peritoneal mast cells

BMMC are differentiated *in vitro* by culturing mouse bone marrow with mast cell specific growth factors, IL-3 and SCF. To control for effects of *in vitro* differentiation, peritoneal mast cells, which mature *in vivo*, can be expanded *ex vivo* to support BMMC results. Mouse peritoneal cells expanded for 7–10 days were treated $\pm 100\mu\text{M}$ Didox (6 hours) and activated for 18 hours by IgE XL. As with BMMC, Didox suppressed IgE-mediated IL-6 and IL-13 secretion, with no effect on MCP-1 (Figure 2A). Didox also reduced peritoneal mast cell degranulation, as measured by CD107a and CD63 staining (Figure 2B). These results further indicate that Didox has selective suppressive effects on cytokine production and confirm that the BMMC results are not a result of *in vitro* differentiation.

3.3. Didox effects are consistent on varied genetic backgrounds

We have previously found that mast cells from the C57BL/6 and 129/SvJ background can vary greatly in their responses to suppressive stimuli, including statins and TGFβ1 [20,27]. Such variability can greatly limit drug efficacy. To ensure the reproducibility of our findings in another genetic background, 129Sv/J BMMC were treated $\pm 100\mu\text{M}$ Didox (6 hours) and IgE-activated for 18 hours. As with C57BL/6 BMMC, Didox inhibited IL-6 and IL-13 secretion, with no effect on MCP-1 (Figure 3). These results suggest that Didox effects are consistent among mice of different genetic backgrounds with many known polymorphisms, increasing the potential utility of Didox in a therapeutic setting.

3.4 Didox effects can be mimicked by antioxidant but not by RNR inhibitor treatment

Didox possesses both RNR inhibitor and antioxidant capabilities [13,15,18,28]. In order to determine if either mechanism is responsible for Didox's suppressive effects, Didox effects were compared to the RNR inhibitor hydroxyurea (HU) and the antioxidant N-acetylcysteine (NAC). HU at concentrations up to 10mM had no effect on IgE-induced mast cell cytokine production. By contrast, NAC (10mM) significantly suppressed IgE-mediated IL-6 and IL-13 secretion (Figure 4A). NAC concentrations of 50mM–1mM had no significant effects (data not shown). Additionally, combining HU and NAC showed no significant change from NAC alone on IL-6 production, and reversed the inhibition of IL-13. Interestingly, both NAC and HU suppressed IgE-mediated degranulation, as measured by CD107a and CD63 (Figure 4B). These results suggest that antioxidant effects likely explain Didox effects on IgE-induced cytokine production, but both its antioxidant and RNR-inhibitory effects may be

tied to suppressing degranulation. It was also important to note that NAC and HU, commonly prescribed clinical drugs, were far less potent than Didox in these assays.

3.5 Didox reduces oxidative stress and upregulates antioxidant genes

Since NAC mimicked Didox suppression of cytokines and degranulation, we wanted to further examine the antioxidant capacity of Didox. First, DCFH-DA fluorescence was measured by flow cytometry as a general indicator of oxidative stress [29]. BMMC were treated $\pm 100 \mu\text{M}$ Didox (6 hours), plated in HBSS with DCFH-DA ($5 \mu\text{M}$), and activated by IgE XL for 2 hours. Didox significantly suppressed fluorescence relative to the H_2O control following activation (Figure 5A). Similarly, expression of superoxide dismutase (SOD1) and catalase were measured to indicate the antioxidant potential of the cells [30]. Didox significantly enhanced catalase and SOD1 expression following 24 hours of IgE-mediated activation (Figure 5B). These data further support the hypothesis that Didox suppresses mast cell activation via antioxidant effects as well as by inducing antioxidant defense mechanisms.

3.6 Didox suppresses FcεRI-mediated NFκB and AP-1 transcriptional activity

IgE XL elicits phosphorylation of signaling proteins and subsequent gene induction by the transcription factors NF κ B and AP-1 [31–33]. We measured Didox effects on NF κ B and AP-1 transcriptional activity, using luciferase reporter vectors. These assays showed that Didox antagonized FceRI-mediated NF κ B- and AP-1-dependent transcription (Figure 6). As these transcription factors are critical for cytokine mRNA induction, this may represent one of the important mechanisms by which Didox exerts its anti-inflammatory effects.

3.7 Didox attenuates IgE-mediated passive systemic anaphylaxis

Our data demonstrate that Didox can suppress IgE-mediated mast cell activation *in vitro*. To test the functional relevance of Didox *in vivo*, we used a model of mast cell-dependent passive systemic anaphylaxis [34,35]. Didox (350mg/kg) was injected IP 6 hrs prior to IgE-mediated PSA. Didox treatment (n=6) attenuated the temperature drop following DNP injection compared with the PBS control (n=5), with significant differences at 30, 45, and 60 minutes (Figure 7). There was no temperature change or difference between groups in the mice that received Didox (n=3) or PBS (n=3) without DNP (data not shown). These results suggest that the suppressive effects of Didox can also be observed *in vivo*.

4. Discussion

Didox was originally created as a cytostatic drug to inhibit cancer cell proliferation by antagonizing RNR [11]. When used to target highly proliferative cells, Didox has extensive activity *in vitro* and *in vivo*. It has also been employed in clinical studies for cancer with minimal toxicity at therapeutic levels [11,14,16,36,37]. In addition to antiproliferative effects, Didox possesses antioxidant and anti-inflammatory activity with significant therapeutic potential to treat diseases associated with oxidative stress and inflammation [17,18,38]. We have recently published Didox suppression of IL-33 mediated mast cell activation [19], however IgE-mediated mast cell function has not been studied. Here we

investigated Didox effects *in vitro* and *in vivo* and the implications they may have for treating allergic inflammation.

Time-course and dose-response experiments showed that Didox optimally suppresses the IgE-mediated mast cell inflammatory response when given 6 hours prior to mast cell activation, with a drug concentration of 100 μ M that does not affect cell viability or receptor expression. Specifically, Didox suppresses mRNA synthesis and protein secretion of inflammatory cytokines and select chemokines, while attenuating mast cell degranulation. These results corroborate previous research, where Didox treatment antagonized pro-inflammatory responses of anti-CD3-activated T cells and LPS-stimulated macrophages in allotransplant and macrophage inflammatory disease models, respectfully [17,18]. Additionally, these data agree with Didox effects on IL-33-mediated mast cell activation [19]. Collectively, these results indicate Didox may have broad anti-inflammatory effects.

Didox was designed to improve on HU-mediated RNR inhibition and reduce unwanted side-effects [11,37,39]. The enzymatic activity of RNR is dependent upon the association of two subunits, R1 and R2, to form an active heterodimeric tetramer. R2 is the iron-binding subunit, while R1 is the substrate-binding subunit. R2 needs a free tyrosyl radical to be active; iron is a cofactor for the generation and stabilization of this free radical. Didox is able to inhibit the association of R1 and R2, and thus suppress RNR activity, through its ability to scavenge free radicals and chelate iron [40,41]. Despite its design as an RNR inhibitor, Didox's free radical scavenger capabilities are important to its broader utility, reducing oxidative stress associated with cardiotoxicity, dementia in HIV patients, and inflammation [13,17,18,42].

To elucidate which of the known Didox biochemical effects explain its ability to suppress IgE-mediated signaling, we used the clinically-approved drugs HU and NAC. The inability of the RNR inhibitor HU to reduce inflammatory cytokines was not surprising, since Fc ϵ RI-mediated signaling is not closely tied to DNA synthesis or proliferation [43–45]. Interestingly, HU suppressed degranulation following activation, albeit at 100-fold lower concentrations than Didox. HU has been used in numerous studies to treat mast cell activation syndrome and mastocytomas, reducing both mast cell load and symptoms of mast cell activation potentially by affecting mediator expression or release [46–48]. However, no studies have examined the effect of HU in healthy, normal mast cells. Our current data suggest that while future studies should examine the mechanisms linking ribonucleotide reductase inhibition to reduced degranulation, they also indicate that Didox may be more efficacious than HU.

In addition to HU, the antioxidant NAC antagonized mast cell activation, but only when used at a 100-fold greater concentration. NAC suppressed cytokine production and degranulation, two pathways that have been linked to oxidative stress and reactive oxygen species production [30,49–51]. Furthermore, Didox suppressed DCFH-DA fluorescence, indicating reduced oxidative stress [29], and Didox increased SOD1 and catalase expression, further increasing antioxidant potential. These results support those observed by Matsebatlela and colleagues, who reported that Didox reduced iNOS expression and NO production, while enhancing SOD1 and catalase activity [18]. Collectively, these data show the potential role

for Didox in modulating oxidative stress in inflammatory diseases, and indicate that Didox may be more potent and efficacious than NAC. Future studies should compared NAC and Didox in other inflammatory disease models, since NAC is in clinical use.

To further address the mechanism of Didox's suppressive effects on mast cell activation, FcεRI receptor expression and downstream transcription factor induction were assessed. We found that Didox had no effect on FcεRI surface expression, and thus concluded that Didox effects must be occurring downstream of receptor activation. The transcription factors NFκB and AP-1 have previously been shown to be critical for FcεRI-mediated cytokine induction [31,33,52]. Lee and Elford have previously shown Didox downregulates NFκB activation in a T-cell model of HIV infection [53,54]. We found that Didox antagonized the FcεRI-induced activity of both transcription factors, confirming and extending this previous report. Because NFκB and AP-1 can be induced by reactive oxygen species [55,56] and Didox suppressed measures of oxidative stress, the antioxidant effects of Didox are a likely mechanism by which Didox suppresses transcription factor activity.

Interestingly, increased antioxidant capacity and suppression of NFκB and AP-1 transcription suggest one explanation for the suppression of inflammatory cytokines, however, our data also showed that Didox is selectively suppressive. We found that Didox has no effect on IgE-mediated MCP-1 expression while studying BMMC from different genetic backgrounds and in peritoneal mast cells. While NFκB- and AP-1-dependent transcription have been linked to production of inflammatory cytokines and chemokines, including MCP-1 [57–59], these results suggest that MCP-1 induction is unique among IgE-induced cytokines. In fact, Metzger's group similarly reported that MCP-1 regulation was unusual in mast cells. While most IgE-induced factors are acutely dependent upon continuous signaling from aggregated receptors, MCP-1 secretion is not [60]. They postulated that MCP-1 may be responsive to weak, intermittent calcium fluxes, which are capable of activating NFκB [61]. Since Didox did not completely abrogate NFκB or AP-1 activity, one possible explanation is that MCP-1 transcription requires a lower threshold of NFκB or AP-1 function. Alternatively, additional transcription factors or post-transcriptional modifications could yield redundant control of MCP-1 secretion, giving resistance to NFκB and AP-1 inhibition. This possibility is supported by the demonstration that MCP-1 expression is regulated by p53 in human keratinocytes [62]. On balance, the inability of Didox to suppress MCP-1 secretion is in keeping with other findings that MCP-1 regulation is unusual among inflammatory cytokines.

Lastly, our data are the first to show that Didox can attenuate inflammation in an *in vivo* model. We found that Didox treatment after IgE sensitization reduced antigen-mediated hypothermia in passive systemic anaphylaxis, a mast cell-dependent model. These data support our cell culture findings and suggest that Didox should be further studied using models of allergic disease, asthma, and inflammation. As a potent analog of the clinically approved drugs HU and NAC, Didox shows significant therapeutic potential.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

BMMC	bone marrow-derived mast cell
XL	cross-linking
IL	interleukin
RNR	ribonucleotide reductase
HU	hydroxyurea
NAC	N-acetylcysteine

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Highlights

- Didox suppresses FcεRI-mediated mast cell degranulation and cytokine production *in vitro*.
- Didox-mediated suppression is partly mimicked by the anti-oxidant N-acetylcysteine, and correlates with increased production of antioxidants, along with reduced oxidative stress.
- Didox has no effect on FcεRI surface expression, but inhibits downstream signaling including the transcriptional activation of NFκB and AP-1.
- Didox suppress IgE-induced passive systemic anaphylaxis *in vivo*.

Figure 1. Didox suppresses IgE XL-induced mast cell function

(A) C57BL/6J bone marrow derived mast cells (BMMC) were pretreated with H₂O (vehicle control) or Didox (100µM) for 24, 6, or 0 hours prior to XL for 18 hours. IL-6 and IL-13 in culture supernatants were measured by ELISA. (B) BMMC were treated with Didox at the indicated concentrations for 6 hours prior to IgE XL for 18 hours. IL-6 and IL-13 in culture supernatants were measured by ELISA. (C) BMMC were treated with Didox (100µM) for 6 hours prior to IgE XL for 18 hours. TNF, MIP-1α, and MCP-1 were measured in culture supernatants by ELISA. (D) BMMC were treated with Didox (100µM) for 6 hours prior to IgE XL for 4 hours. Total RNA was extracted and analyzed by RT-qPCR for IL-6 and

GAPDH. IL-6 expression was determined relative to GAPDH. (E) BMMC were treated with Didox (100 μ M) for 6 hours prior to IgE XL for 10 minutes. Degranulation was determined by CD107a and CD63 expression via flow cytometry using APC α -CD107a and PE-anti-mouse CD63. Data are means $\pm$ SEM of 2 (B,C,D) or 3 (A,E) independent experiments, each performed using 3 independent BMMC populations. * p < 0.05, ** p < .01, *** p < .001 compared to matching vehicle control. NSD= no significant difference.

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Figure 2. Didox selectively suppresses FcεRI-mediated cytokine production by peritoneal mast cells

(A) C57BL/6J peritoneal mast cells were treated with Didox (100μM) for 6 hours prior to IgE XL for 18 hours. IL-6, IL-13, and MCP were measured in culture supernatants by ELISA. (B) C57BL/6J peritoneal mast cells were treated with Didox (100μM) for 6 hours prior to IgE XL for 10 minutes. Degranulation was determined by CD107a and CD63 expression via flow cytometry using APC α-CD107a and PE-antimouse CD63. Data are means ± SEM of 3 independent experiments, each performed using 3 independent BMMC populations. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ compared to matching vehicle control. NSD= no significant difference.

Figure 3. Didox selectively suppresses IgE XL-induced cytokine secretion by 129/SvJ BMMC
 129Sv/J BMMC were pretreated with H_2O (vehicle control) or Didox (at the indicated concentrations) for 6 hours prior to IgE XL for 18 hours. Cytokines were measured in culture supernatants by ELISA. Data are means $\pm$ SEM of 4 independent experiments, each performed with 3 independent BMMC populations. * $p < 0.05$, ** $p < .01$, *** $p < .001$ compared to matching vehicle control. NSD= no significant difference.

Figure 4. Antioxidant, but not RNR inhibition, mimics Didox effects

C57BL/6J BMMC were pretreated with H₂O (vehicle control), Didox (100μM), HU (10 mM), or NAC (10 mM) for 6 hours prior to IgE XL for (A) 18 hours to collect cytokines for ELISA analysis or (B) 10 minutes for degranulation analysis by flow cytometry.

Degranulation was determined by CD107a and CD63 expression using APC α-CD107a and PE-anti- mouse CD63. Data are means ± SEM of 3 (A) or 2 (B) independent experiments, each performed with 3 independent BMMC populations. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ compared to matching vehicle control.

Figure 5. Didox suppresses oxidative stress and upregulates catalase and SOD1

(A) C57BL/6J BMMC were treated with Didox (100 μ M) for 6 hours prior to IgE XL for 2 hrs. DCFH-DA was added at 5 μ M 30 minutes prior to activation, and fluorescence was analyzed on the FITC channel by flow cytometry. (B) BMMC were treated with Didox (100 μ M) for 6 hours prior to IgE XL for 24 hrs. Total RNA was extracted and analyzed by RT-qPCR for catalase and SOD1 expression, relative to actin. Data are means $\pm$ SEM of 2 (B) or 3 (A) independent experiments, each performed using 3 independent BMMC populations. * p < 0.05, ** p < .01, *** p < .001 compared to matching vehicle control.

Figure 6. Didox suppresses NFκB and AP-1 transcriptional activity downstream of FcεRI
 NFκB (A) or AP-1 (B) firefly luciferase and Renilla control luciferase were transfected into C57BL/6J BMMC. After overnight sensitization with IgE, H₂O (vehicle control) or Didox (100μM) was added for 6 hours prior to IgE XL for 2 hours. Relative luminescence (firefly/renilla) was determined with a luminometer. Data are means ± SEM of 2 independent experiments, each performed with 3 independent BMMC populations. * $p < 0.05$, ** $p < .01$, *** $p < .001$ compared to matching vehicle control.

Figure 7. Didox attenuates IgE-mediated passive systemic anaphylaxis

C57BL/6J were sensitized overnight with one anti-DNP IgE (50 µg) intraperitoneal (IP) injection. The next day, mice were IP injected with PBS (5 mice) or Didox (350 mg/kg, 6 mice) 6 hours prior to an IP injection of PBS or DNP-HSA (100 mg) to elicit anaphylaxis. Core body temperature was measured for 2 hours. * $p < 0.05$, ** $p < .01$, *** $p < .001$ compared to matching vehicle control.