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Research paper

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

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ABSTRACT

Mast cell activation via the high-affinity IgE receptor (FcεRI) 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-1a (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 FcεRI expression or cell viability, suggesting it inhibits signaling required for inflammatory cytokine production. In support of this, we found that Didox reduced FcεRI-mediated AP-1 and NFκB 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.

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

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

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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 100 mM, briefly sonicated, and filter sterilized (0.45 mm syringe filter, Cell Treat). Didox was added to cultures at a final concentration of 100 μ M 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 Fig. 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 $\geq 90\%$ Fc ϵ RI⁺ 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 $\sim 85\%$ Fc ϵ RI⁺ 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 0.5 mg/mL anti-DNP mouse IgE (k isotype). Next, cells were washed and resuspended at 1×10^6 cells/mL with IL-3 and SCF (10 ng/mL). Crosslinking (XL) was induced by the addition of DNP-HSA (50 ng/mL) for 18 h. Didox or vehicle control (DEPC water) was added for 6 h prior to IgE activation unless otherwise stated.

2.5. ELISA

Following Didox treatment and IgE activation for 18 h, 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 h for IL-6 analysis or 24 h 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 NanoDrop™ 1000 UV-vis Spectrophotometer (ThermoScientific, Waltham, MA). For mRNA detection, cDNA was synthesized using qScript™ 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'-GATGACATCAAGAA GGTGGTG-3'; reverse: 5'-GCTGTAGCCAAATTCGTTGTC-3'), SOD1 (forward: 5'-CGGATGAAGAGAGGCATGTT-3'; reverse: 5'-CACCTTTGCCAAGTCATCT-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 min followed by 40 cycles of 95 °C for 15 s, 55 °C for 30 s and 60 °C for 1 min. 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 h 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 (100 mg/mL), EDTA, and propidium iodide (200 mg/mL) for 2–3 h 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-IgG2 a (clone RTK2758, Biolegend) and PE rat IgG2 b (BD Pharmingen)), 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 min activation, cells were washed and analyzed by flow cytometry. For analysis of oxidative stress, cells were treated with Didox for 6 h, then resuspended in Hank's buffered saline solution (HBSS) + 2',7'-Dichlorofluorescein Diacetate (DCFH-DA, 5 μ M, Millipore) $\pm$ Didox for 30 min at 37 °C. Cells were then activated with DNP (50 ng/mL) for 2 h, 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 min 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 [hRluc/TK] encoding the luciferase gene from *Renilla reniformis* under the HSV-TK promoter, and either pGL4.32[luc2 p/NF κ B RE/Hygro] or pGL4.44[luc2 p/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 hRluc:NF κ B/AP1. The protocol was as previously described [22] using the Amara Nucleofector (Lonza, Allendale, NJ). All experiments were performed 48 h 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).

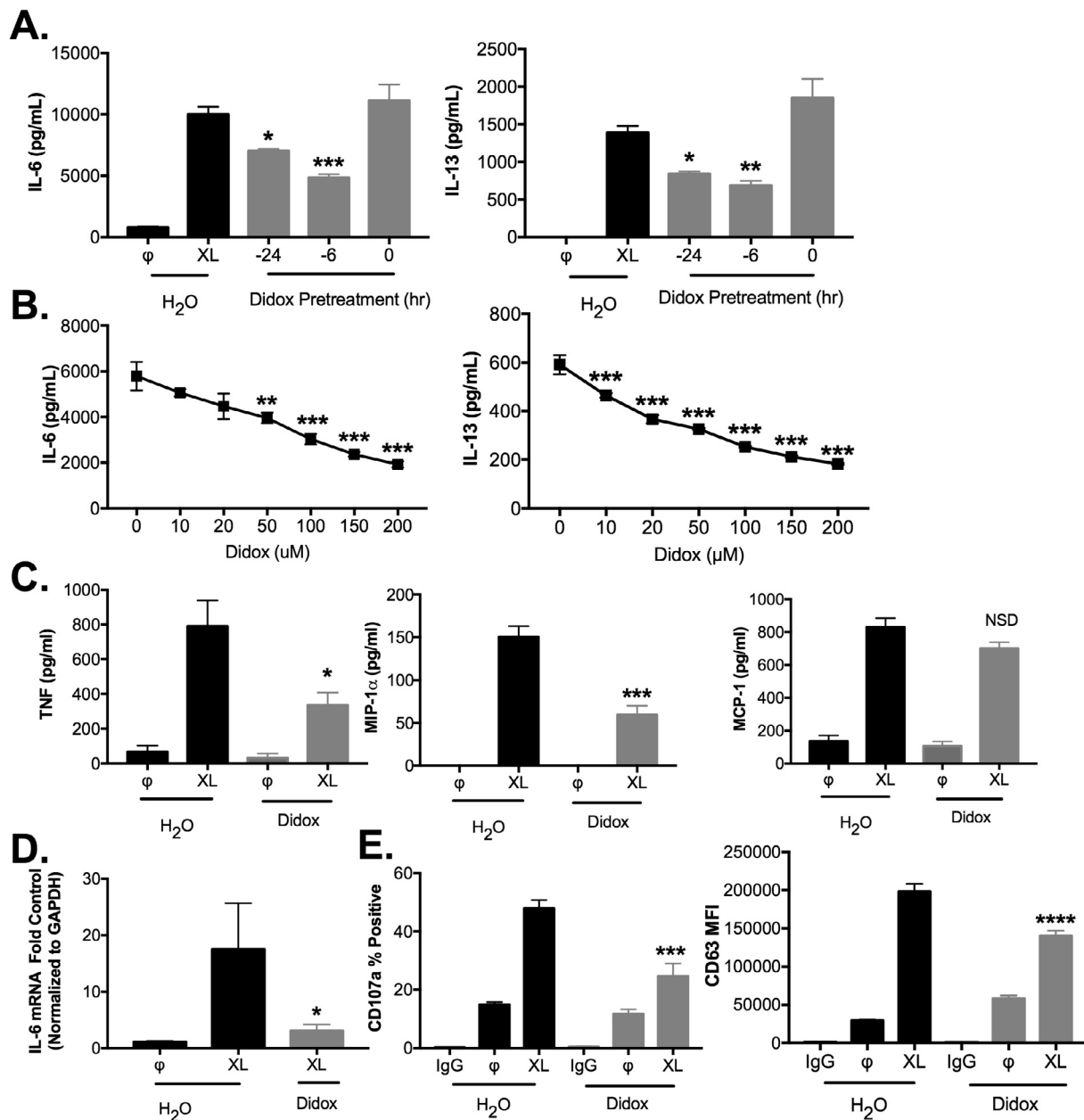


Fig. 1. Didox suppresses IgE XL-induced mast cell function. (A) C57BL/6J bone marrow derived mast cells (BMMC) were pretreated with H₂O (vehicle control) or Didox (100 μM) for 24, 6, or 0 h prior to XL for 18 h. IL-6 and IL-13 in culture supernatants were measured by ELISA. (B) BMMC were treated with Didox at the indicated concentrations for 6 h prior to IgE XL for 18 h. IL-6 and IL-13 in culture supernatants were measured by ELISA. (C) BMMC were treated with Didox (100 μM) for 6 h prior to IgE XL for 18 h. TNF, MIP-1α, and MCP-1 were measured in culture supernatants by ELISA. (D) BMMC were treated with Didox (100 μM) for 6 h prior to IgE XL for 4 h. 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 μM) for 6 h prior to IgE XL for 10 min. Degranulation was determined by CD107a and CD63 expression via flow cytometry using APC α-CD107a and PE-anti- mouse CD63. Data are means ± SEM of 2 (B,C,D) or 3 (A,E) 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.

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 h 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 h (Physitemp Instruments).

2.10. Statistical analyses

For Figs. 1–6, a one-way analysis of variance (ANOVA) was used to detect differences between groups. For Fig. 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

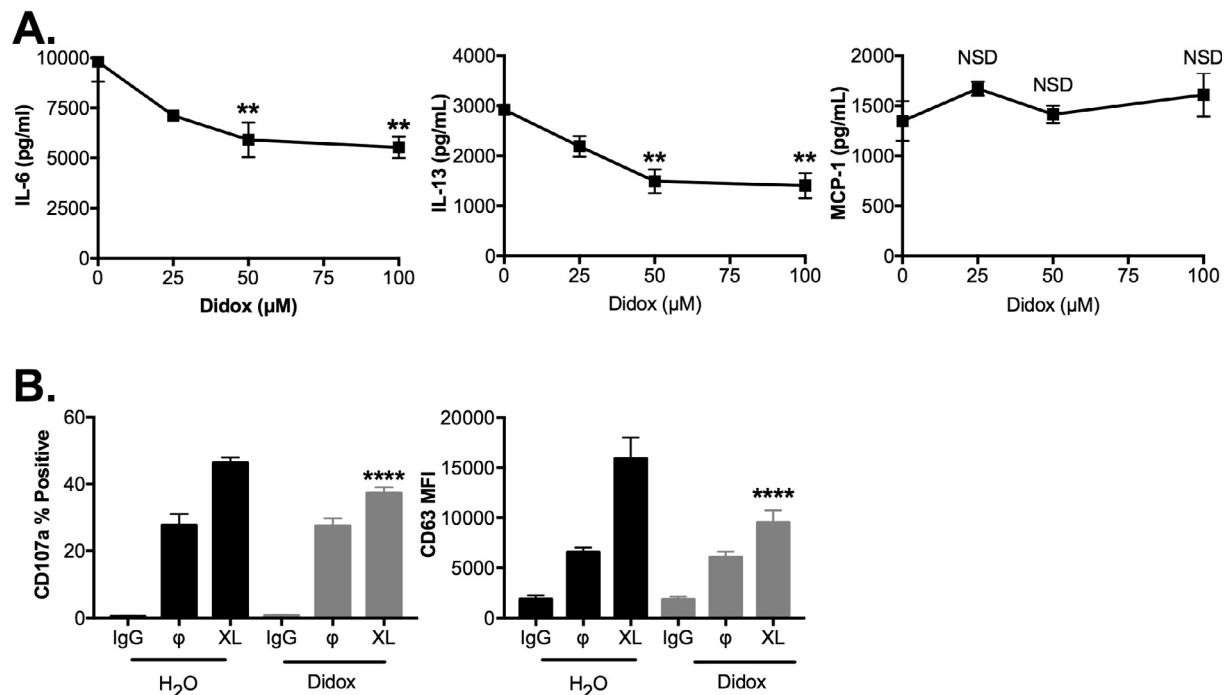


Fig. 2. Didox selectively suppresses FcεRI-mediated cytokine production by peritoneal mast cells. (A) C57BL/6J peritoneal mast cells were treated with Didox (100 μM) for 6 h prior to IgE XL for 18 h. IL-6, IL-13, and MCP were measured in culture supernatants by ELISA. (B) C57BL/6J peritoneal mast cells were treated with Didox (100 μM) for 6 h prior to IgE XL for 10 min. Degranulation was determined by CD107a and CD63 expression via flow cytometry using APC α-CD107a and PE-anti-mouse 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.

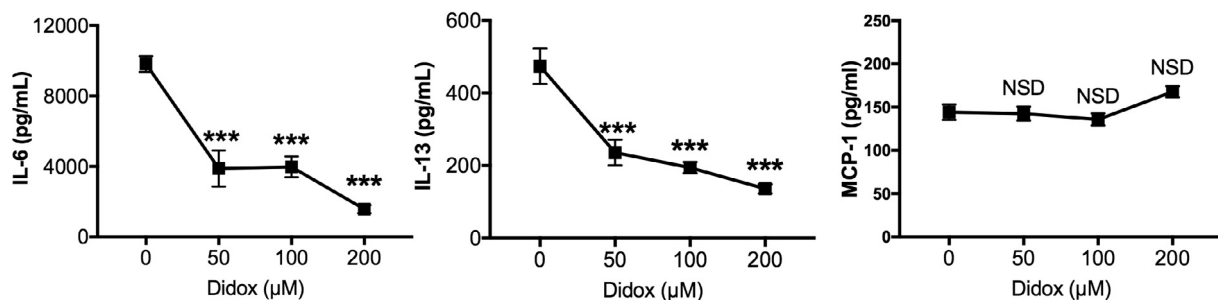


Fig. 3. Didox selectively suppresses IgE XL-induced cytokine secretion by 129/SvJ BMMC. 129Sv/J BMMC were pretreated with H₂O (vehicle control) or Didox (at the indicated concentrations) for 6 h prior to IgE XL for 18 h. Cytokines were measured in culture supernatants by ELISA. Data are means ± SEM of 4 independent experiments, each performed with 3 independent BMMC populations. **p* < 0.05, ***p* < 0.01, ****p* < 0.001 compared to matching vehicle control. NSD = no significant difference.

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 ± 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 h prior to antigen (Fig. 1A). To determine dose-dependent effects, IgE-primed BMMC were treated ± Didox at multiple doses for 6 h and activated via IgE cross-linking (XL). Significant suppression was achieved at concentrations ranging from 50 to 200 μM (Fig. 1B). The treatment parameters of 100 μM Didox for 6-h and 24-h (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-h 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-h Didox treatment

(Supplementary Fig. 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 100 μM Didox treatment for 6 h.

In addition to IL-6 and IL-13 (Fig. 1A and B), Didox suppressed TNF and MIP-1a (CCL3) secretion, but had no significant effect on MCP-1 (CCL2) (Fig. 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 (Fig. 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 ± 100 μM Didox for 6 h, followed by antigen-mediated cross-linking for 10 min. Increases in percent CD107a+ and CD63 MFI were significantly suppressed among Didox-treated versus vehicle-treated

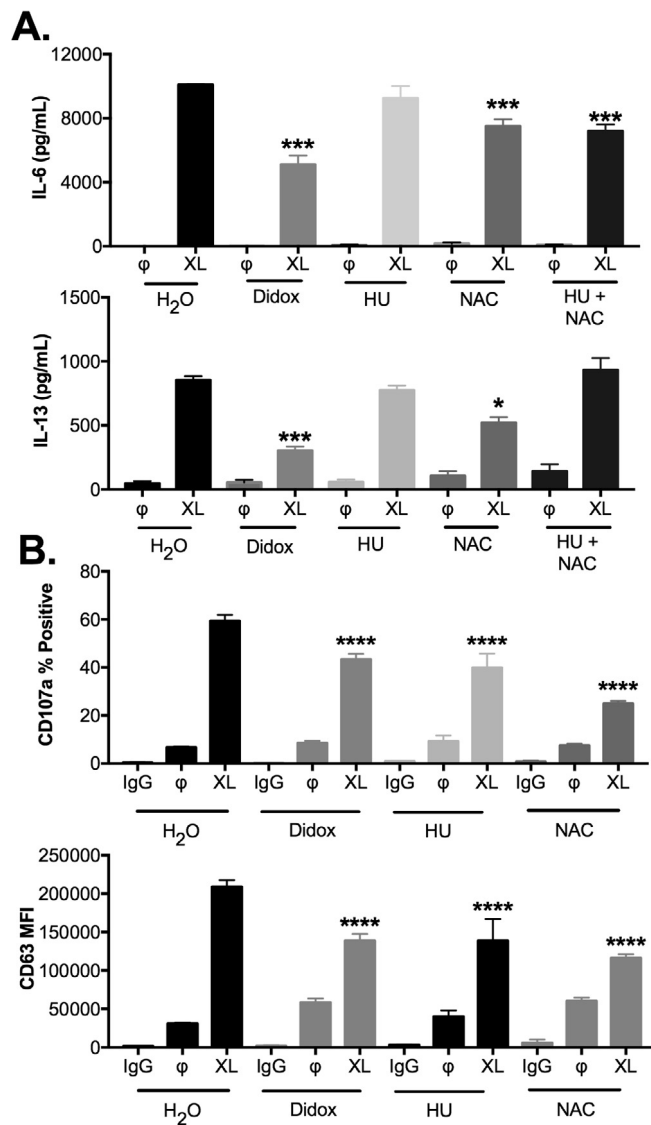


Fig. 4. Antioxidant, but not RNR inhibition, mimics Didox effects. C57BL/6J BMMC were pretreated with H₂O (vehicle control), Didox (100 μ M), HU (10 mM), or NAC (10 mM) for 6 h prior to IgE XL for (A) 18 h to collect cytokines for ELISA analysis or (B) 10 min for degranulation analysis by flow cytometry. Degranulation was determined by CD107a and CD63 expression using APC α -CD107a and PE-anti-mouse CD63. Data are means $\pm$ 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.

BMMC (Fig. 1E). Collectively these results show Didox inhibits both the early and late phases of mast cell activation.

3.2. Didox suppresses Fc ϵ RI-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 μ M Didox (6 h) and activated for 18 h by IgE XL. As with BMMC, Didox suppressed IgE-mediated IL-6 and IL-13 secretion, with no effect on MCP-1 (Fig. 2A). Didox also reduced peritoneal mast cell degranulation, as measured by CD107a and CD63 staining (Fig. 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.

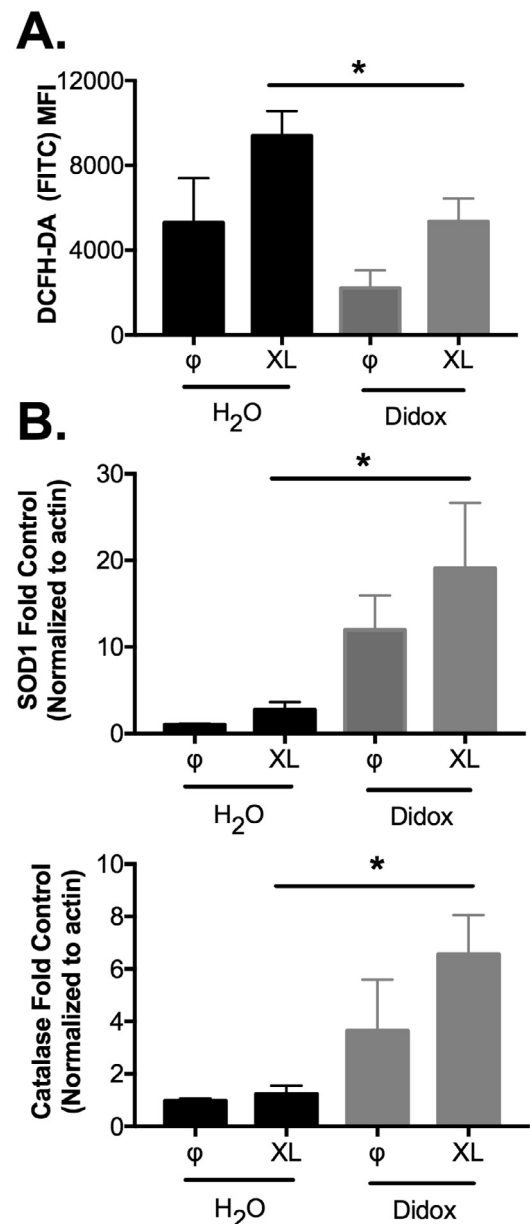


Fig. 5. Didox suppresses oxidative stress and upregulates catalase and SOD1. (A) C57BL/6J BMMC were treated with Didox (100 μ M) for 6 h prior to IgE XL for 2 h. DCFH-DA was added at 5 μ M 30 min prior to activation, and fluorescence was analyzed on the FITC channel by flow cytometry. (B) BMMC were treated with Didox (100 μ M) for 6 h prior to IgE XL for 24 h. 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 < 0.01, *** p < 0.001 compared to matching vehicle control.

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 TGF β 1 [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 μ M Didox (6 h) and IgE-activated for 18 h. As with C57BL/6 BMMC, Didox inhibited IL-6 and IL-13 secretion, with no effect on MCP-1 (Fig. 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.

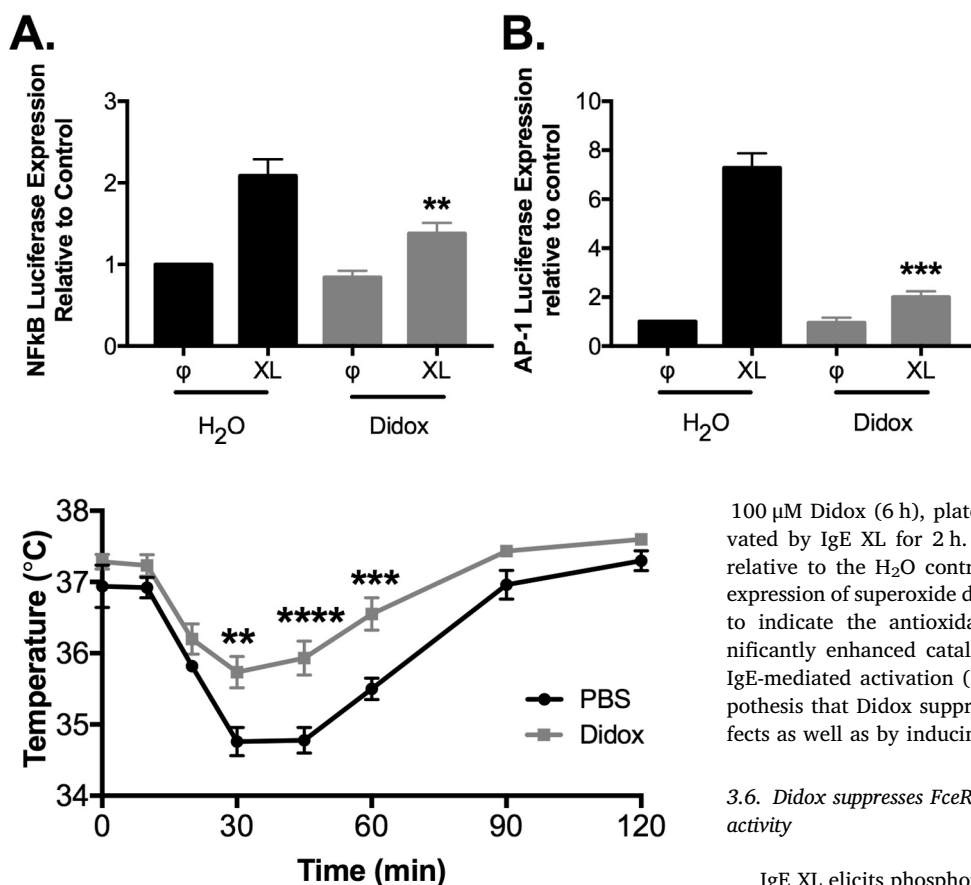


Fig. 7. Didox attenuates IgE-mediated passive systemic anaphylaxis. C57BL/6J were sensitized overnight with one anti-DNP IgE (50 µg) intraperitoneal (IP) injection. The next day, mice were IP injected with PBS (5 mice) or Didox (350 mg/kg, 6 mice) 6 h prior to an IP injection of PBS or DNP-HSA (100 mg) to elicit anaphylaxis. Core body temperature was measured for 2 h. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ compared to matching vehicle control.

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 10 mM had no effect on IgE-induced mast cell cytokine production. By contrast, NAC (10 mM) significantly suppressed IgE-mediated IL-6 and IL-13 secretion (Fig. 4A). NAC concentrations of 50 mM–1 mM 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 (Fig. 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 ±

Fig. 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 (100 µM) was added for 6 h prior to IgE XL for 2 h. Relative luminescence (firefly/re-nilla) was determined with a luminometer. Data are means ± SEM of 2 independent experiments, each performed with 3 independent BMMC populations. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ compared to matching vehicle control.

100 µM Didox (6 h), plated in HBSS with DCFH-DA (5 µM), and activated by IgE XL for 2 h. Didox significantly suppressed fluorescence relative to the H₂O control following activation (Fig. 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 h of IgE-mediated activation (Fig. 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 NFκB and AP-1 [31–33]. We measured Didox effects on NFκB and AP-1 transcriptional activity, using luciferase reporter vectors. These assays showed that Didox antagonized FcεRI-mediated NFκB- and AP-1-dependent transcription (Fig. 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 (350 mg/kg) was injected IP 6 h 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 min (Fig. 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 h 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, respectively [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 Matsebatiela 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 compare 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, Fc ϵ RI receptor expression and downstream transcription factor induction were assessed. We found that Didox had no effect on Fc ϵ RI surface expression, and thus concluded that Didox effects must be occurring downstream of receptor activation. The transcription factors NF κ B and AP-1 have previously been shown to be critical for Fc ϵ RI-mediated cytokine induction [31,33,52]. Lee and Eelford have previously shown Didox downregulates NF κ B activation in a T-cell model of HIV infection [53,54]. We found that Didox antagonized the Fc ϵ RI-induced activity of both transcription factors, confirming and

extending this previous report. Because NF κ B 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 NF κ B 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 NF κ B- 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 NF κ B [61]. Since Didox did not completely abrogate NF κ B or AP-1 activity, one possible explanation is that MCP-1 transcription requires a lower threshold of NF κ B or AP-1 function. Alternatively, additional transcription factors or post-transcriptional modifications could yield redundant control of MCP-1 secretion, giving resistance to NF κ B 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.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at <http://dx.doi.org/10.1016/j.cellimm.2017.09.008>.

References

- [1] J. Kalesnikoff, S.J. Galli, New developments in mast cell biology, *Nat. Immunol.* 9 (2008) 1215–1223.
- [2] K. Amin, The role of mast cells in allergic inflammation, *Respir. Med.* 106 (2012) 9–14.
- [3] O.T. Burton, H.C. Oettgen, Beyond immediate hypersensitivity: evolving roles for IgE antibodies in immune homeostasis and allergic diseases, *Immunol. Rev.* 242 (2011) 128–143.
- [4] S.J. Galli, J.R. Gordon, B.K. Wershil, Cytokine production by mast cells and basophils, *Curr. Opin. Immunol.* 3 (1991) 865–872.
- [5] F.D. Finkelman, Anaphylaxis: lessons from mouse models, *J. Allergy Clin. Immunol.* 120 (2007) 506–515.
- [6] D.D. Metcalfe, R.D. Peavy, A.M. Gilfillan, Mechanisms of mast cell signaling in anaphylaxis, *J. Allergy Clin. Immunol.* 124 (2009) 639–646.
- [7] B.A. Sayed, M.E. Walker, M.A. Brown, Cutting edge: mast cells regulate disease severity in a relapsing-remitting model of multiple sclerosis, *J. Immunol.* 186 (2011) 3294–3298.
- [8] D.M. Lee, Mast cells: a cellular link between autoantibodies and inflammatory arthritis, *Science* 297 (2002) 1689–1692.
- [9] J. Sun, G.K. Sukhova, P.J. Wolters, M. Yang, S. Kitamoto, P. Libby, et al., Mast cells promote atherosclerosis by releasing proinflammatory cytokines, *Nat. Med.* 13

- (2007) 719–724.
- [10] V.H. Secor, W.E. Secor, C.A. Gutekunst, M.A. Brown, Mast cells are essential for early onset and severe disease in a murine model of multiple sclerosis, *J. Exp. Med.* 191 (2000) 813–822.
 - [11] H.L. Elford, G.L. Wampler, B.V. Riet, New ribonucleotide reductase inhibitors with antineoplastic activity, *Cancer Res.* 39 (1979) 844–851.
 - [12] M.S. Inayat, D. Chendil, M. Mohiuddin, H.L. Elford, V.S. Gallicchio, M.M. Ahmed, Didox (a novel ribonucleotide reductase inhibitor) overcomes Bcl-2 mediated radiation resistance in prostate cancer cell line PC-3, *Cancer Biol. Ther.* 1 (2002) 539–545.
 - [13] J. Turchan, C.B. Pocerich, C. Gairola, A. Chauhan, G. Schifitto, D.A. Butterfield, et al., Oxidative stress in HIV demented patients and protection ex vivo with novel antioxidants, *Neurology* 60 (2003) 307–314.
 - [14] J. Carmichael, B.M. Cantwell, K.A. Mannix, D. Veale, H.L. Elford, R. Blackie, et al., A phase I and pharmacokinetic study of didox administered by 36 hour infusion. The cancer research campaign phase I/II clinical trials committee, *Br. J. Cancer* 61 (1990) 447–450.
 - [15] N. Raj, S. Kumar, T. Hideshima, K. Ishitsuka, H. Yasui, S. Chhetri, et al., Didox, a ribonucleotide reductase inhibitor, induces apoptosis and inhibits DNA repair in multiple myeloma cells, *Br. J. Haematol.* 135 (2006) 52–61.
 - [16] R.D. Rubens, S.B. Kaye, M. Soukop, C.J. Williams, M.H. Brampton, A.L. Harris, Phase II trial of didox in advanced breast cancer. Cancer research campaign phase I/II clinical trials committee, *Br. J. Cancer.* 64 (1991) 1187–1188.
 - [17] M.S. Inayat, I.S. El-Amouri, M. Bani-Ahmad, H.L. Elford, V.S. Gallicchio, O.R. Oakley, Inhibition of allogeneic inflammatory responses by the ribonucleotide reductase inhibitors, didox and trimidox, *J. Inflamm. (Lond.)* 7 (2010) 43.
 - [18] T.M. Matsebatlela, A.L. Anderson, V.S. Gallicchio, H. Elford, C.D. Rice, 3,4-Dihydroxy-benzohydroxamic acid (Didox) suppresses pro-inflammatory profiles and oxidative stress in TLR4-activated RAW264.7 murine macrophages, *Chem. Biol. Interact.* 233 (2015) 95–105.
 - [19] H.L. Caslin, J.J.A. McLeod, A.J. Spence, A.A. Qayum, E.M. Kolawole, M.T. Taruselli, et al., Didox (3,4-dihydroxybenzohydroxamic acid) suppresses IL-33-induced cytokine production in primary mouse mast cells, *Cell. Immunol.* (2017) 1–7.
 - [20] E.M. Kolawole, J.J.A. McLeod, V. Ndaw, D. Abebayehu, B.O. Barnstein, T. Faber, et al., Fluvastatin suppresses mast cell and basophil ige responses: genotype-dependent effects, *J. Immunol.* 196 (2016) 1461–1470.
 - [21] D. Abebayehu, A.J. Spence, A.A. Qayum, M.T. Taruselli, J.J.A. McLeod, H.L. Caslin, et al., Lactic acid suppresses IL-33-mediated mast cell inflammatory responses via hypoxia-inducible factor-1 α -dependent miR-155 suppression, *J. Immunol.* 197 (2016) 2909–2917.
 - [22] A. Paranjape, O. Chernushevich, A.A. Qayum, A.J. Spence, M.T. Taruselli, D. Abebayehu, et al., Dexamethasone rapidly suppresses IL-33-stimulated mast cell function by blocking transcription factor activity, *J. Leukoc. Biol.* 100 (2016) 1395–1404.
 - [23] A. Nieto-Patlán, M. Campillo-Navarro, O. Rodríguez-Cortés, S. Muñoz-Cruz, I. Wong-Baeza, S. Estrada-Parra, et al., Recognition of *Candida albicans* by Dectin-1 induces mast cell activation, *Immunobiology* 220 (2015) 1093–1100.
 - [24] M. Köberle, S. Kaesler, W. Kempf, F. Wölbing, T. Biedermann, Tetraspanins in mast cells, *Front. Immunol.* 3 (2012) 106.
 - [25] F. Hennesdorf, S. Florian, A. Jakob, K. Baumgärtner, K. Sonneck, A. Nordheim, et al., Identification of CD13, CD107a, and CD164 as novel basophil-activation markers and dissection of two response patterns in time kinetics of IgE-dependent upregulation, *Cell Res.* 15 (2005) 325–335.
 - [26] M. Zhu, J. Zou, T. Li, S.A. O'Brien, Y. Zhang, S. Ogden, et al., Differential roles of phospholipase D proteins in Fc ϵ R1-mediated signaling and mast cell function, *J. Immunol.* 195 (2015) 4492–4502.
 - [27] J. Fernando, T.W. Faber, N.A. Pullen, Y.T. Falanga, E.M. Kolawole, C.A. Oskertizian, et al., Genotype-dependent effects of TGF- β 1 on mast cell function: targeting the Stat5 pathway, *J. Immunol.* 191 (2013) 4505–4513.
 - [28] S. Madlener, C. Illmer, Z. Horvath, P. Saiko, A. Losert, I. Herbacek, et al., Gallic acid inhibits ribonucleotide reductase and cyclooxygenases in human HL-60 promyelocytic leukemia cells, *Cancer Lett.* 245 (2007) 156–162.
 - [29] B. Kalyanaraman, V. Darley-Usmar, K.J.A. Davies, P.A. Dennerly, H.J. Forman, M.B. Grisham, et al., Measuring reactive oxygen and nitrogen species with fluorescent probes: challenges and limitations, *Free Rad. Biol. Med.* 52 (2012) 1–6.
 - [30] E.M. Conner, M.B. Grisham, Inflammation, free radicals, and antioxidants, *Nutrition* 12 (1996) 274–277.
 - [31] D.L. Marquardt, L.L. Walker, Dependence of mast cell IgE-mediated cytokine production on nuclear factor-kappaB activity, *J. Allergy Clin. Immunol.* 105 (2000) 500–505.
 - [32] M.J. Nadler, S.A. Matthews, H. Turner, J.P. Kinet, Signal transduction by the high-affinity immunoglobulin E receptor Fc epsilon R1: coupling form to function, *Adv. Immunol.* 76 (2000) 325–355.
 - [33] A. Lorentz, I. Klopp, T. Gebhardt, M.P. Manns, S.C. Bischoff, Role of activator protein 1, nuclear factor- κ B, and nuclear factor of activated T cells in IgE receptor-mediated cytokine expression in mature human mast cells, *J. Allergy Clin. Immunol.* 111 (2003) 1062–1068.
 - [34] R.T. Strait, S.C. Morris, M. Yang, X.-W. Qu, F.D. Finkelman, Pathways of anaphylaxis in the mouse, *J. Allergy Clin. Immunol.* 109 (2002) 658–668.
 - [35] J. Kalesnikoff, S.J. Galli, Anaphylaxis: mechanisms of mast cell activation, *Chemical Immunology and Allergy, KARGER, Basel*, 2010, pp. 45–66.
 - [36] L.R. Sumpter, M.S. Inayat, E.E. Yost, W. Duvall, E. Hagan, C.N. Mayhew, et al., In vivo examination of hydroxyurea and the novel ribonucleotide reductase inhibitors trimidox and didox in combination with the reverse transcriptase inhibitor abacavir: suppression of retrovirus-induced immunodeficiency disease, *Antiviral Res.* 62 (2004) 111–120.
 - [37] D. Veale, J. Carmichael, B.M. Cantwell, H.L. Elford, R. Blackie, D.J. Kerr, et al., A phase 1 and pharmacokinetic study of didox: a ribonucleotide reductase inhibitor, *Br. J. Cancer.* 58 (1988) 70–72.
 - [38] H.L. Elford, B. van't Riet, G.L. Wampler, A.L. Lin, R.M. Elford, Regulation of ribonucleotide reductase in mammalian cells by chemotherapeutic agents, *Adv. Enzyme Regul.* 19 (1980) 151–168.
 - [39] B. van't Riet, G.L. Wampler, H.L. Elford, Synthesis of hydroxy- and amino-substituted benzohydroxamic acids: inhibition of ribonucleotide reductase and anti-tumor activity, *J. Med. Chem.* 22 (1979) 589–592.
 - [40] T. Szekeres, P. Saiko, M. Fritzer-Szekeres, B. Djavan, W. Jäger, Chemopreventive effects of resveratrol and resveratrol derivatives, *Ann. N. Y. Acad. Sci.* 1215 (2011) 89–95.
 - [41] M.F. Szekeres, L. Novotny, A. Vachalkova, G. Findenig, H.L. Elford, T. Szekeres, Iron binding capacity of Didox (3,4-Dihydroxybenzohydroxamic acid) and Amidox (3,4-Dihydroxybenzamidoxime) new inhibitors of the enzyme ribonucleotide reductase, *Life Sci.* 61 (1997) 2231–2237.
 - [42] A.M. Al-Abd, F.A. Al-Abbasi, G.F. Asaad, A.B. Abdel-Naim, Didox potentiates the cytotoxic profile of doxorubicin and protects from its cardiotoxicity, *Eur. J. Pharmacol.* 718 (2013) 361–369.
 - [43] M. Rådinger, B.M. Jensen, H.S. Kuehn, A. Kirshenbaum, A.M. Gilfillan, Generation, isolation, and maintenance of human mast cells and mast cell lines derived from peripheral blood or cord blood, *Curr. Protoc. Immunol.* (2010) Unit-Un37.
 - [44] P.N. Weraahra, L.M. Glode, F.G. La Rosa, M.S. Lucia, E.D. Crawford, K. Easterday, et al., Proliferative tumor doubling times of prostatic carcinoma, *Prostate Cancer* 2011 (2011) 1–7.
 - [45] G.I. Evan, K.H. Vousden, Proliferation, cell cycle and apoptosis in cancer, *Nature* 411 (2001) 342–348.
 - [46] L.B. Afrin, Utility of hydroxyurea in mast cell activation syndrome, 2 (2013) 1–1.
 - [47] F. Mellouli, S. Chouaibi, N. Dhoubi, M. Ouederni, M. Ben Khaled, S. Abbes, et al., Effectiveness and acceptance of hydroxyurea in the treatment of severe forms of sickle cell disease: a prospective study of 65 cases, *Tunis Med.* 91 (2013) 544–550.
 - [48] K.M. Rassnick, R. Al-Sarraf, D.B. Bailey, J.D. Chretien, B. Phillips, C.H. Zwhalen, Phase II open-label study of single-agent hydroxyurea for treatment of mast cell tumours in dogs, *Vet. Comp. Oncol.* 8 (2010) 103–111.
 - [49] E. Naik, V.M. Dixit, Mitochondrial reactive oxygen species drive proinflammatory cytokine production: Fig. 1, *J. Exp. Med.* 208 (2011) 417–420.
 - [50] M.A. Chelombitko, A.V. Fedorov, O.P. Ilyinskaya, R.A. Zinovkin, B.V. Chernyak, Role of reactive oxygen species in mast cell degranulation, *Biochem. Moscow* 81 (2016) 1564–1577.
 - [51] X. Gan, D. Xing, G. Su, S. Li, C. Luo, M.G. Irwin, et al., Propofol attenuates small intestinal ischemia reperfusion injury through inhibiting NADPH oxidase mediated mast cell activation, *Oxid. Med. Cell. Longevity* 2015 (2015) 1–15.
 - [52] R. Sibilano, B. Frossi, C.E. Pucillo, Mast cell activation: a complex interplay of positive and negative signaling pathways, *Eur. J. Immunol.* 44 (2014) 2558–2566.
 - [53] R. Lee, P. Beauparlant, H. Elford, P. Ponka, J. Hiscott, Selective inhibition of I kappaB alpha phosphorylation and HIV-1 LTR-directed gene expression by novel antioxidant compounds, *Virology* 234 (1997) 277–290.
 - [54] H. Elford, R. Lee, J. Turchan, V. Gallicchio, M. Ussery, J. Hiscott, et al., The virtues of unique ribonucleotide reductase inhibitors didox and trimidox for retrovirus therapy, *Antiviral Res.* 74 (2007) A65–A66.
 - [55] G. Gloire, E. Dejardin, J. Piette, Extending the nuclear roles of I κ B kinase subunits, *Biochem. Pharmacol.* (2006).
 - [56] U. Vollgraf, M. Wegner, C. Richter-Landsberg, Activation of AP-1 and nuclear factor-kappaB transcription factors is involved in hydrogen peroxide-induced apoptotic cell death of oligodendrocytes, *J. Neurochem.* 73 (1999) 2501–2509.
 - [57] K.B. Ahn, J.H. Jeon, S.-S. Kang, D.K. Chung, C.-H. Yun, S.H. Han, IgE in the absence of allergen induces the expression of monocyte chemoattractant protein-1 in the rat basophilic cell-line RBL-2H3, *Mol. Immunol.* 62 (2014) 114–121.
 - [58] A. Ueda, Y. Ishigatsubo, T. Okubo, T. Yoshimura, Transcriptional regulation of the human monocyte chemoattractant protein-1 gene, *J. Biol. Chem.* 272 (1997) 31092–31099.
 - [59] A. Alonso, Stimulation of Fc gamma R receptors induces monocyte chemoattractant protein-1 in the human monocyte cell line THP-1 by a mechanism involving I kappa B-alpha degradation and formation of p50/p65 NF-kappa B/Rel complexes, *Int. Immunol.* 12 (2000) 547–554.
 - [60] Z.J. Liu, H. Haleem-Smith, H. Chen, H. Metzger, Unexpected signals in a system subject to kinetic proofreading, *Proc. Natl. Acad. Sci. U.S.A.* 98 (2001) 7289–7294.
 - [61] R.E. Dolmetsch, K. Xu, R.S. Lewis, Calcium oscillations increase the efficiency and specificity of gene expression, *Nature.* 392 (1998) 933–936.
 - [62] K. Hacke, B. Rincon-Orozco, G. Buchwalter, S.Y. Siehler, B. Wasylyk, L. Wiesmüller, et al., Regulation of MCP-1 chemokine transcription by p53, *Mol. Cancer.* 9 (2010) 82.