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Combination of ebselen and hydrocortisone substantially reduces nitrogen mustard-induced cutaneous injury

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ABSTRACT

The purpose of the present study was to investigate the vesicant countermeasure effects of hydrocortisone (HC) and ebselen (EB-1), administered as monotherapy or as a combination treatment. The mouse ear vesicant model (MEVM) was utilized and test doses of HC (0.016, 0.023, 0.031, 0.047, 0.063, 0.125 or 0.250 mg/ear), EB-1 (0.125, 0.187, 0.250, 0.375 or 0.500 mg/ear) or the combination of HC + EB-1 were topically applied at 15 min, 4 h and 8 h after nitrogen mustard exposure. Ear punch biopsies were obtained 24 h after mechlorethamine (HN2) exposure. Compared to control ears, ear tissues exposed topically to HN2 (0.500 μmol/ear) presented with an increase in ear thickness, vesication, TUNEL fluorescence and expression of matrix metalloproteinase 9 (MMP-9) and inducible nitric oxide synthase (iNOS). In contrast, HN2 exposed ears treated topically with EB-1 showed a significant decrease in morphometric thickness and vesication vs. HN2 alone. Ear tissues exposed to HN2 and then treated with HC also demonstrated reductions in morphometric thickness and vesication. Combination treatment of HC + EB-1 was found to be the most effective at reducing HN2-induced ear edema and vesication. The combination also dramatically decreased HN2-mediated cutaneous expression of iNOS and MMP-9 and decreased HN2-induced TUNEL staining. Taken together, our study demonstrates that the combination of HC + EB-1 is an efficacious countermeasure to HN2.

Introduction

Vesicant chemical warfare agents (v-CWAs) cause dermal-epidermal junction (DEJ) disruption and cutaneous blistering accompanied by severe inflammation. In addition to v-CWAs, several nitrogen mustard chemotherapy drugs such as mechlorethamine (HN2), bendamustine, and ifosfamide are associated with DEJ disruption resulting from extravasation reactions (EVRs), which can occasionally occur when the chemotherapeutic drug escapes from veins into the surrounding cutaneous tissue, causing undesirable effects ranging from discomfort, tissue swelling and localized inflammation to blistering (vesication) and ulcer formation (Bertelli, 1995; Ener et al., 2004; Cassagnol and McBride, 2009). Aside from v-CWAs, such as Lewisite or sulfur mustard, and the nitrogen mustard chemotherapy drugs mentioned above, certain natural products act as DEJ disruptors, including environmental toxins found in the venom of certain snakes or insects (Gutiérrez et al., 2018; Herrera et al., 2018; Rucavado et al., 1998; Torbeck et al., 2014; Wijerathne, 2017). DEJ disruptors remain a public health concern primarily because, except for British anti-Lewisite for organoarsenicals, there are no broad-spectrum anti-blistering agents currently available; thus, treatments of DEJ disruption remain palliative in nature.

An important goal of our laboratory over the past decade has been to identify countermeasures that reduce the toxicity of HN2, the prototype DEJ disruptor. In the past, we utilized an *in vitro* approach to pursue this goal (Pino and Billack, 2008; Pino et al., 2014; Lulla et al., 2013; Hardej and Billack, 2007). More recently, we have employed the mouse ear vesicant model (MEVM) to study skin responses to HN2 and have discovered that the organoselenium compound ebselen (EB-1; Fig. 1, left panel) reduces tissue swelling associated with HN2 exposure (Lulla et al., 2014). Moreover, the glucocorticoid hydrocortisone (HC; Fig. 1, right panel) has been previously reported to reduce tissue swelling associated with cutaneous exposure to HN2 (Lulla et al., 2014) and sulfur mustard (Casillas et al., 2000). The potential countermeasure activity of topically applied HC administered in combination with EB-1 has not yet been evaluated in the MEVM. We hypothesize that the pharmacologic effect of the combination will be more pronounced than that of either agent separately.

In the present study, we used the MEVM to investigate cutaneous responses to HN2. We observed and confirmed that 24 hr after a single

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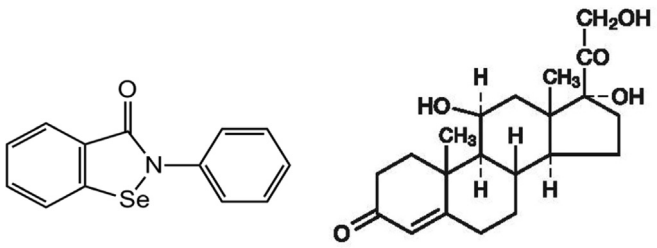

Fig 1. Chemical structures of ebselen (EB-1, left panel) and hydrocortisone (HC, right panel).

topical application of HN2, mouse ear skin exhibited massive immune cell infiltration, necrosis, increased TUNEL activity (an indicator of DNA injury), an upregulation of the tissue injury-associated matrix metalloproteinase 9 (MMP-9) (Cuffari et al., 2018; Kumar et al., 2015; Tumu et al., 2020; Wormser et al., 2002) and an increased tissue presence of the proinflammatory enzyme inducible nitric oxide synthase (iNOS) (Biyashev et al., 2020; Composto et al., 2016; Kumar et al., 2015; Tumu et al., 2020). MMP-9 is a proteolytic enzyme capable of degrading extracellular matrix proteins such as denatured collagen (gelatin) and collagen types IV, V, VII, IX and X (van der Jagt et al., 2006). iNOS is involved in several inflammatory and pathological conditions, especially in regulating, via the synthesis of nitric oxide, pathogen clearance and acute inflammation in wound healing (Malone-Povolny et al., 2019).

Therefore, to evaluate the nitrogen mustard countermeasure potential of the combination of an organoselenium (EB-1) with a glucocorticoid (HC), we exposed mouse ears to a vesicating dose of HN2 and then, at 15 min, 4 hr and 8 hr post exposure, we administered EB-1 and HC via the topical route, either alone or together. Then, as described above, the ear tissues were harvested and examined for edema, vesication and expression of iNOS and MMP-9 24 hr following the initial HN2 exposure. We observed that the combination was indeed more effective than either agent alone as a post-treatment after HN2 exposure and, importantly, was able to completely suppress vesication. All in all, the present study represents an important contribution to the chemical countermeasures field.

Materials and methods

Test compounds

Dimethyl sulfoxide (DMSO) was obtained from J.T. Baker (Philipsburg, NJ; Cat# 67–68-5). Mechlorethamine hydrochloride (HN2) was purchased from Pfaltz & Bauer (Waterbury, CT; Cat # 55–86-7). DMSO served as a vehicle for both HN2 and the test compounds or was used as a vehicle control. EB-1 was kindly provided by Dr. Magdalena Pietka-Ottlik (Wroclaw University of Science and Technology, Poland). Hydrocortisone (HC) was purchased from Alfa Aesar (Ward Hill, MA; Cat# A16292).

Chemicals, reagents, kits and other materials

Isoflurane (Cat # 029405) was purchased from Henry Schein (Dublin, OH). Permount was purchased from Fisher Scientific (Fairlawn, NJ; Cat# SP15-500). Slides and cover glasses were purchased from VWR International (Radnor, PA; Cat# 16004–386 and Cat # 48382-136, respectively). Eosin (Cat # CA95057-848), hematoxylin (Cat # CA95057-844), xylene (Cat # CA95057-822), histology grade 100% ethanol (Cat # CA95057-828) and Paraplast X-tra (Cat # 15159-486 -1 kg) were purchased from VWR International (West Chester, PA). Neutral buffered formalin (1:10 dilution, already diluted) (Cat # 23-245-685) was purchased from Fisher Scientific (Nazareth, PA). Vectastain ABC Rabbit IgG Kit (Cat # PK-6101) and Antigen Unmasking Solution (Citrate Based) (Cat # H-3300) were both purchased from Vector Laboratories (Burlingame, CA). ApopTag Fluorescein In situ Apoptosis Detection kit was purchased from EMD Millipore Corporation (Billerica, MA; Cat # S7110). Methyl Green, zinc chloride salt was purchased from VWR International (Salon, OH; Cat #: 97061-398). Proteinase K was purchased from VWR international (Salon, OH; Cat # 97062-238). DAPI/Antifade solution and Propidium iodide/Antifade solution were purchased from EMD Millipore Corporation (Temecula, CA; Cat # S7113 and Cat # S7112). ProLong Gold antifade reagent was purchased from ThermoFisher Scientific (Eugene, OR; Cat # P36930). Phosphate buffered saline (PBS) (10X) liquid concentrate was obtained from EMD Millipore (Gibbstown, NJ; Cat # 6505-OP). Tris Buffered Saline (TBS) (10X) was purchased from VWR International (West Chester, PA; Cat # 10128-548). Tween-20 was purchased from VWR International (Solon, OH; Cat # 97062-332). 30% Hydrogen Peroxide (H_2O_2) was purchased from VWR International (Mississauga, ON, Cat # BDH7690-1). The 100% n-butanol was purchased from EMD Millipore (Billerica, MA; Cat # BX1777-6).

Animals

Outbred male Swiss Webster mice (25–30 g) were purchased from Taconic farms (Germantown, NY). All mice were kept and maintained in the AAALAC-accredited Animal Care Center at St. John's University (Queens, NY). All animals were housed in groups of 2–8 per cage in temperature and humidity regulated rooms with 12 h - day and 12 h - night cycles. Animals were allowed to adjust to the new environment for at least 2–3 days before use. The protocol for this research was approved by the Institutional Animal Care and Use Committee (IACUC) of St. John's University and the animals were cared for in accordance with the guidelines established by the U.S. Department of Agriculture (USDA).

Mouse ear vesicant model (MEVM)

When mouse ear skin is exposed to HN2, robust cutaneous responses including hyperplasia, neutrophil infiltration, DEJ disruption (vesication) and epidermal necrosis are observed within a 24 hr period (Casillas et al., 1997; Casillas et al., 2000; Cuffari et al., 2018; Dachir et al., 2002; Tumu et al., 2020). In our studies, DMSO was used as the vehicle for both HN2 (vesicant) and the test compounds (EB-1 and HC).

Test solutions and reagents

(a) HN2 solution: mechlorethamine hydrochloride in the amount of 0.0192 g (molecular weight: 192.52) was dissolved in 1 ml of DMSO to obtain a 0.100 M of HN2. When 5 µl of this solution is applied to the inner surface of a mouse ear, it is equal to a HN2 dose of 0.5 µmol/ear. This topical dose was used here because it has previously been found by our laboratory to robustly induce the formation of microblisters in Swiss Webster mice (Cuffari et al, 2018; Lulla et al., 2014; Tumu et al., 2020) and C57BL mice (Sparavalo, 2013).

(b) Ebselen: Five microliter (5 µl) volumes of 0.025 mg/µl, 0.037 mg/µl, 0.050 mg/µl, 0.075 mg/µl and 0.100 mg/µl EB-1 test solutions were applied to the ear (inner, ventral surface) to obtain the test doses of 0.125 mg/ear, 0.187 mg/ear, 0.250 mg/ear, 0.375 mg/ear and 0.500 mg/ear, respectively.

(c) Hydrocortisone: HC test solutions were applied to the ear (inner, ventral surface) at test doses of 0.015 mg/ear, 0.023 mg/ear, 0.031 mg/ear, 0.047 mg/ear, 0.063 mg/ear, 0.125 mg/ear and 0.250 mg/ear, respectively.

(d) HC + EB-1: To prepare combination treatments, stock solutions of HC were mixed together with stock solutions of EB-1 in a 1:1 ratio. Hence, 10 μl of 0.012 mg/μl of HC when mixed together with 10 μl of 0.100 mg/μl EB-1 yielded a combination stock with 0.006 mg/μL of HC and 0.050 mg/μL of EB-1. When this premixed solution was applied as a 5 μl volume to the inner mouse ear, topical doses of 0.031 mg/ear of HC and 0.250 mg/ear of EB-1 were delivered to the tissue. In a similar manner, the other EB-1 test solutions described above were premixed with HC test solutions so that when 5 μl of the premixed stock was applied to the ear, test doses of 0.031 mg/ear HC + 0.187 mg/ear EB-1, 0.047 mg/ear HC + 0.187 mg/ear EB-1, 0.047 mg/ear HC + 0.250 mg/ear EB-1 or 0.250 mg/ear HC + 0.500 mg/ear EB-1 were delivered to the ear tissue.

(e) Other reagents. Vehicle: 5 μl of DMSO were applied to the ears that served as controls in vehicle control tissues. Buffered formalin solution (1:10 dilution): Neutral buffered formalin (8 ml) was used for fixation of each ear punch. Dehydration alcohol solutions: Histology grade dehydration ethanol 100% was diluted with distilled and deionized water to obtain 30%, 60%, 70% and 95% ethanol concentrations. Hematoxylin staining solution: Ready-made hematoxylin solution purchased from VWR was used to stain the nuclei of the tissue sections. Eosin staining solution: Ready-made eosin solution purchased from VWR was used to stain the cytoplasm of the tissue sections.

Experimental design

24 h study: Three treatments with a single test countermeasure at 15 min, 4 h and 8 h post-HN2 exposure: Wild type, male Swiss Webster mice weighing 25–30 g were separated into groups. The right ears were treated with a 5 μl volume of HN2 (0.500 μmol/ear), while the left ears served as test controls.

To test the effectiveness of EB-1, a 5 μl volume of EB-1 solutions that yield the doses of either 0.125 mg/ear, 0.187 mg/ear, 0.250 mg/ear, 0.375 mg/ear or 0.500 mg/ear dose of EB-1 was applied to the right ears of the mice in the respective groups, three times, at 15 min, 4 hr and 8 hr after the HN2 exposure. Both the HN2-exposed and the control ears were treated three times with the respective doses of EB-1 at 15 min, 4 hr and 8 hr following the initial HN2 treatment on the right ear.

To test the effectiveness of HC, a 5 μl volume of HC solutions that yield the doses of 0.015 mg/ear, 0.023 mg/ear, 0.031 mg/ear, 0.047 mg/ear, 0.063 mg/ear, 0.125 mg/ear and 0.250 mg/ear HC was applied to the right ears of the mice in the respective groups, three times, at 15 min, 4 hr and 8 hr after the HN2 exposure. Both the HN2-exposed and the control ears were treated three times with the respective doses of HC at 15 min, 4 hr and 8 hr following the HN2 treatment.

To test the effectiveness of HC + EB-1 combination treatments, a 5 μl volume of premixed solutions that yielded doses of 0.031 mg/ear HC + 0.187 mg/ear EB-1, 0.031 mg/ear HC + 0.250 mg/ear EB-1, 0.047 mg/ear HC + 0.187 mg/ear EB-1, 0.047 mg/ear HC + 0.250 mg/ear EB-1, or 0.250 mg/ear HC + 0.500 mg/ear EB-1 was applied to the right ears of the mice in the respective groups, three times, at 15 min, 4 hr and 8 hr after the HN2 exposure. Both the HN2 exposed and the control ears were treated three times with the respective doses of HC + EB-1 at 15 min, 4 hr and 8 hr following the HN2 treatment.

Twenty-four hours after the exposure, animals were euthanized using carbon dioxide (CO_2) and ear tissue samples were collected using 8 mm biopsy punches. The ear samples were then transferred to 20 ml vials with 10 ml neutral buffered formalin for 24 hr before dehydration, embedding in paraffin, tissue sectioning and H&E staining as described previously (Tumu et al., 2020).

Morphometric analysis

Ear thickness was measured as described previously (Tumu et al., 2020). In brief, the H & E stained sections were measured under a total magnification of 40× with software calibrated measurements. Thickness of the ear tissues were measured by drawing nine perpendicular lines from one side of the tissue to the other side for each section, and the average length was determined. The average thickness values obtained from two duplicate slides for each ear were used in the morphometric analysis.

Histopathological evaluation

A Zeiss Axio Scope A1 microscope equipped with Axiocam 506 color camera with Zeiss Zen 2.3 software was used for obtaining light microscopy images of immunohistochemistry (IHC) and H & E stained tissues. Stained tissues were evaluated for vesication (DEJ disruption). Two duplicate slides for each ear were used and scoring for vesication was performed by four independent investigators. A positive score for vesication was assessed when 4 or more of the eight scores for a particular treatment were positive for at least one epidermal detachment. The study was completely blinded. Since all the slides were coded, none of the investigators knew the details of the sections until they were decoded at the end of the study.

Fluorescein in situ terminal deoxynucleotidyl transferase (TdT) dUTP nick end labeling (TUNEL) assay for apoptosis Detection

The assay was performed as described by the manufacturer (EMD Millipore Corporation, Billerica, MA; Cat # S7110). A Leica DMIL inverted microscope equipped with SLF-130-LX ScopeLED fluorescence system and Luminera Infinity 3 mono camera with Infinity capture and Infinity analyze 6.5.4 was used for obtaining fluorescence microscopy images.

Immunohistochemistry (IHC)

IHC for MMP-9 and iNOS was carried out as described previously (Tumu et al., 2020). The primary antibodies used here included a 1:100 dilution of either rabbit polyclonal anti-MMP9 antibody (Cat # ab38898; Abcam, Cambridge, MA) or rabbit polyclonal anti-iNOS antibody (ab3523; Abcam, Cambridge, MA).

Statistical analysis

All the results were reported as mean ± SEM for 6 samples. Statistical significance for morphometric analysis was tested between groups using a one-way ANOVA followed by Newman – Keuls multiple comparison post hoc analysis and GraphPad Prism® version 5.0 software.

Results

Effects of EB-1 and HC, each administered alone or in combination, on HN2-induced tissue edema

First, HN2 was used to induce ear edema. To this end, the inner surface of the right ears of male Swiss Webster mice were treated with HN2 (0.5 μmol/ear). Left ears served as test controls and were not exposed to HN2. Control and HN2 treated mouse ears were then subjected to three treatments of either DMSO or EB-1, HC or the combination of EB-1 + HC (right ears) at 15 min, 4 hr and 8 hr after HN2 exposure. EB-1 test doses included 0.125, 0.187, 0.250, 0.375 and 0.500 mg/ear. HC test doses included 0.016, 0.023, 0.031, 0.047, 0.063, 0.125 and 0.250 mg/ear. Combination treatment test doses

included 0.031 mg/ear HC + 0.187 mg/ear EB-1; 0.031 mg/ear HC + 0.250 mg/ear EB-1; 0.047 mg/ear HC + 0.187 mg/ear EB-1; 0.047 mg/ear HC + 0.250 mg/ear EB-1 and 0.250 mg/ear HC + 0.500 mg/ear EB-1). Ear biopsies were obtained 16 hr after the last intervention treatment (24 hr after the initial HN2 exposure). The morphometric thickness of untreated naive ear tissues was 242.6 μm and the test control DMSO-treated left ear biopsies exhibited a morphometric thickness of 253.5 μm. On the other hand, ear tissues topically exposed to HN2 followed by three treatments of DMSO showed a nearly threefold increase in morphometric thickness as compared to the test controls, indicating that HN2 caused severe swelling of the ear tissues (Table 1).

HN2 exposed ear tissues treated with EB-1 at all test doses showed statistically significant decreases in morphometric ear thickness compared to HN2 exposed ears treated with DMSO (Table 1, Supplemental Fig. 1, Panel B). HN2 exposed ear tissues treated with HC (0.023, 0.031, 0.047 and 0.125 mg/ear) showed a statistically significant reduction in morphometric thickness when compared to HN2 exposed ears treated with DMSO (Table 1, Supplemental Fig. 1, Panel A). Lastly, as compared to HN2 exposed ears treated thrice with DMSO, HN2 exposed ear tissues treated thrice with the combination of HC (0.031 mg/ear) and EB-1 (0.250 mg/ear) reduced the morphometric ear thickness by ~ 39%, proving to be the most effective combination treatment among all those tested (Table 1, Supplemental Fig. 1, Panel C).

Effects of EB-1, HC and the combination of the two on HN2-induced changes is ear tissue histology

Light micrographs revealed that ear sections obtained from animals treated with EB-1 (0.250 mg/ear) were similar to the ear sections treated with vehicle (DMSO) (Fig. 2, Panels A and B), suggesting that EB-1 alone did not cause any visible injury of the ear sections. Similarly, ears treated with HC (0.031 mg/ear) alone (Fig. 2, Panel C) or the combination of HC (0.031 mg/ear) and EB-1 (0.25 mg/ear), alone (Fig. 2, Panel D) did not exert any visible injury of the ear sections. On the other hand, topical exposure to HN2 (0.500 μmol/ear) followed by three treatments of DMSO showed severe tissue swelling and dermal-epidermal detachment (vesication) compared to vehicle only treated ear tissues (Fig. 2, compare Panel E to Panel A). HN2-exposed ear tissues treated thrice with EB-1 (0.250 mg/ear) at 15 min, 4 hr and 8 hr after HN2 treatment appeared less edematous compared to the HN2 treated ear tissues (Fig. 2, compare Panel F to Panel E). HN2-exposed ear tissues treated thrice with HC (0.031 mg/ear) also appeared less edematous compared to the HN2 (Fig. 2, compare Panel G to Panel E). Lastly, compared to HN2 exposed ears treated thrice with DMSO, HN2 exposed ear tissues treated thrice with the combination of HC (0.031 mg/ear) and EB-1 (0.250 mg/ear) showed a dramatic reduction in edema compared to HN2-exposed tissues (Fig. 2, compare Panel H to Panel E). These histology results for the combination treatment were confirmed by morphometric thickness analysis ($P < 0.001$; Fig. 3).

Table 1
Assessment of edema in the right (HN2 treated) ear tissues obtained from male Swiss Webster mice 24 h after topical exposure to 0.5 μmol/ear HN2 followed by DMSO, EB-1, HC or the combination of HC + EB-1.

Treatment (mg/ear)[a]	Morphometric thickness (μm)[b] *(Mean ± SEM of (n) of samples).*	N
Untreated	242.6 ± 4.1	6
DMSO	253.5 ± 4.7	6
HN2 + DMSO	691.3 ± 20.1***	6
HN2 + EB-1 (0.125 mg/ear)	575.7 ± 15.1***, ####	6
HN2 + EB-1 (0.187 mg/ear)	541.4 ± 22.4***, ####	6
HN2 + EB-1 (0.250 mg/ear)	579.1 ± 16.2***, ####	6
HN2 + EB-1 (0.375 mg/ear)	562.9 ± 6.7***, ####	6
HN2 + EB-1 (0.500 mg/ear)	560.2 ± 9.9***, ####	6
HN2 + HC (0.016 mg/ear)	602.6 ± 22.5***	6
HN2 + HC (0.023 mg/ear)	551.0 ± 26.2***, ##	6
HN2 + HC (0.031 mg/ear)	550.6 ± 8.5***, ##	6
HN2 + HC (0.047 mg/ear)	534.2 ± 27.4***, ###	6
HN2 + HC (0.063 mg/ear)	596.5 ± 30.6***	6
HN2 + HC (0.125 mg/ear)	548.6 ± 29.1***, ##	6
HN2 + HC (0.250 mg/ear)	599.5 ± 25.0***	9
HN2 + HC (0.031) + EB-1 (0.250) mg/ear	424.1 ± 37.1***, ###	6

[a]Right ears were exposed to HN2 and treated with DMSO vehicle or specific doses of, EB-1, HC or the combination of HC + EB-1.
[b]Morphometric thickness analysis of H & E-stained ear tissue sections obtained from male Swiss Webster mice 24 h after HN2 treatment. Statistical differences were significant between DMSO and the HN2 treated groups at ***$p < 0.001$. Statistical differences were also significant between the HN2 + DMSO group and the groups of HN2 exposed ears treated with specific doses of EB-1, HC or HC + EB-1 at ###$p < 0.001$, ##$p < 0.01$.

Effects of EB-1, HC and the combination of the two on HN2-induced vesication

In the absence of HN2, no vesication was observed in any of the mouse ears treated with DMSO only (Table 2). Exposure to HN2 at the dose of 0.5 μmol/ear alone followed by three treatments with DMSO resulted in a 100% incidence of vesication observed at 24 hr (N = 6; see Table 2). EB-1 applied to mouse ears at test doses of 0.125, 0.187, 0.250, 0.375 and 0.500 mg/ear after HN2 exposure reduced the incidence of vesication to 0.0%, 16.7%, 16.7%, 33.3% and 50.0%, respectively. HC applied at test doses of 0.016, 0.023, 0.031, 0.047, 0.063, 0.125 and 0.250 mg/ear after HN2 reduced the incidence of vesication to 33.3%, 33.3%, 33.3%, 16.7%, 0.0%, 0.0% and 33.3%, respectively (Table 2). Lastly, the combination of HC (0.031 mg/ear) and EB-1 (0.250 mg/ear) applied topically completely abrogated ear tissue vesication by HN2. These data are summarized in Table 2.

TUNEL staining

Fluorescent TUNEL micrographs revealed that ear sections obtained from animals treated with EB-1 (0.250 mg/ear) were similar to the ear sections treated with vehicle (DMSO) (Fig. 4, Panels A and B). Similarly, ears treated with HC alone (Fig. 4, Panel C) or the combination of HC and EB-1 alone (Fig. 4, Panel D) did not exert any TUNEL fluorescence. On the other hand, tissues topically exposed to HN2 (0.500 μmol/ear) followed by three treatments of DMSO exhibited strong TUNEL fluorescence in both the exposed epithelium as well as the dermis when compared to vehicle only treated ear tissues (Fig. 4, compare Panel E to Panel A). HN2-exposed ear tissues treated three times with EB-1 (0.250 mg/ear) at 15 min, 4 hr and 8 hr after HN2 treatment showed less TUNEL fluorescence when compared to the HN2 treated ear tissues (Fig. 4, compare Panel F to Panel E). HN2-exposed ear tissues treated thrice with HC (0.031 mg/ear) also showed reduced TUNEL fluorescence, particularly in the dermis (Fig. 4, compare Panel G to Panels A and E). Lastly, compared to HN2 exposed ears treated thrice with DMSO, considerably less TUNEL fluorescence was observed for HN2 exposed ear tissues treated thrice with the combination of HC (0.031 mg/ear) and EB-1 (0.250 mg/ear) (Fig. 4, compare Panel H to Panel A).

TUNEL + cells were counted in all tissue sections and found to decrease in order of magnitude in the following pattern, HN2-exposed tissues > HN2-exposed tissues treated with EB-1 or HC alone > HN2-exposed tissues treated with the combination of HC + EB-1 (Supplementary Fig. 2, Panel A). Indeed the effect of the combination of HC + EB-1 on TUNEL + cell counts on HN2-treated tissues, as with edema, appears to be an additive effect.

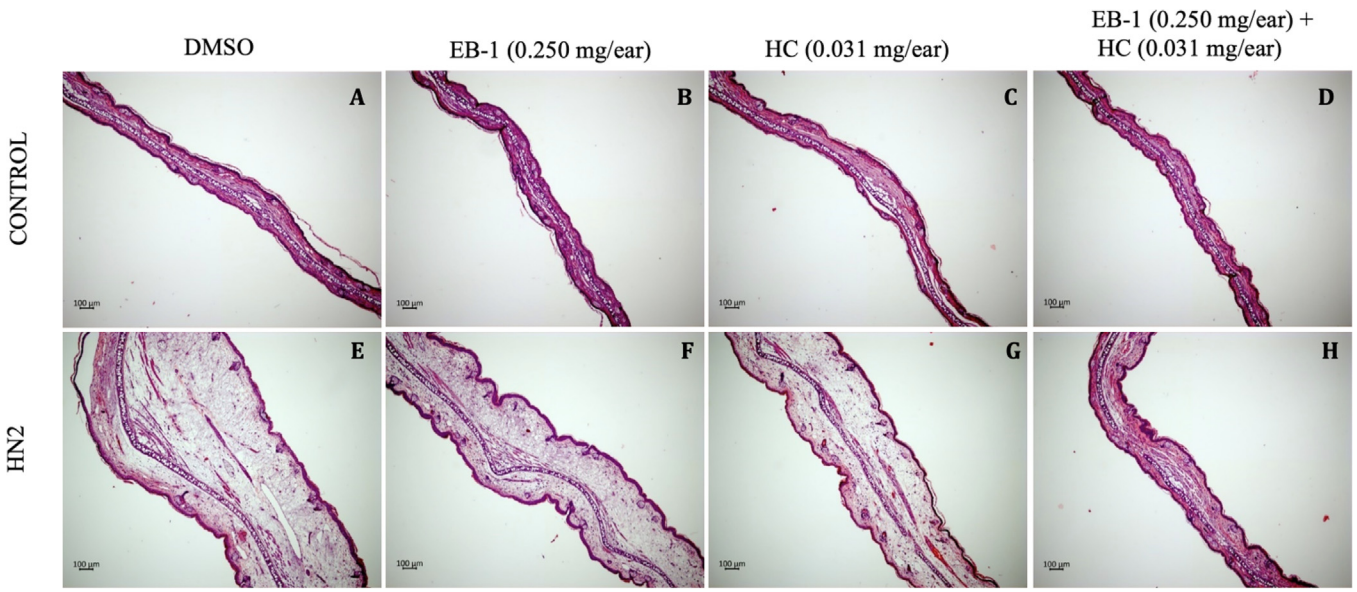

Fig. 2. Representative light micrographs of H & E stained ear tissue sections obtained from male Swiss Webster mice H&E staining of ear tissues obtained 24 h after topical administration of (A) vehicle (DMSO), (B) EB-1 (0.25 mg/ear), (C) HC (0.031 mg/ear) or (D) HC (0.031) + EB-1 (0.250) mg/ear applied at 15 min, 4 h and 8 h; or with HN2 (0.5 μmol/ear) followed by three treatments (3X) of (E) vehicle (DMSO) (F) EB-1 (0.25 mg/ear), (G) HC (0.031 mg/ear) or (H) HC (0.031) + EB-1 (0.250) mg/ear applied at 15 min, 4 h and 8 h. Magnification used was 50×. Bars on the micrograph represent a length of 100 μm.

IHC for MMP-9

Light micrographs of IHC for MMP-9 revealed that ear sections obtained from animals treated with EB-1 at a test dose of 0.250 mg/ear (Fig. 5, Panel B) were similar to the ear sections treated with vehicle (DMSO) (Fig. 5, Panel A), indicating that EB-1 at a test dose of 0.250 mg/ear alone did not affect tissue expression of MMP-9. Similarly, ears treated with HC alone (Fig. 5, Panel C) or the combination of HC and EB-1 alone (Fig. 5, Panel D) did not exhibit MMP-9 expression. On the other hand, expression of MMP-9 was observed in mouse ear tissues exposed to HN2, on both the HN2 treated side (Fig. 5, Panel E) of the dermis, as well as the contralateral side of the dermis (untreated side, Fig. 5, Panel I). Ear tissues treated three times with EB-1 at a test dose of 0.250 mg/ear at 15 min, 4 hr and 8 hr after HN2 treatment (Fig. 5, Panels F and J) showed decreased tissue expression of MMP-9 when compared to HN2-exposed ear tissues treated thrice with DMSO (Fig. 5, Panels E and I). HN2-exposed ear tissues treated thrice with HC (0.031 mg/ear) also showed less HN2-induced MMP-9 tissue expression (Fig. 5, compare Panels G and K to Panels E and I). Lastly, compared to HN2 exposed ears treated thrice with DMSO, the tissue expression of MMP-9 observed in HN2 exposed ear tissues treated thrice with the combination of HC (0.031 mg/ear) + EB-1 (0.250 mg/ear) was dramatically reduced and looked similar to ear sections treated with vehicle alone (Fig. 5, compare Panels H and L to Panel A). MMP9 + cells were counted in all tissue sections and found to decrease in order of magnitude in the following pattern, HN2-exposed tissues > HN2-exposed tissues treated with EB-1 or HC alone or in combination (Supplementary Fig. 2, Panel B). In other words, the effect of the combination of HC + EB-1 on the quantity of MMP9 + cells in HN2-treated tissues did not appear to be stronger than the reduction produced by either agent administered alone.

IHC for iNOS

Light micrographs showing tissue expression of iNOS revealed that ear sections obtained after treatment with EB-1 at a test dose of 0.250 mg/ear (Fig. 6, Panel B) were similar to those observed for ear sections following treatment with vehicle alone (DMSO) (Fig. 6, Panel A), indicating that EB-1 at a test dose of 0.250 mg/ear alone did not affect tissue expression of iNOS. Similarly, ears treated with HC alone (Fig. 6, Panel C) or the combination of HC and EB-1 alone (Fig. 6, Panel D) did not exhibit iNOS expression in the ear tissues, at least at 24 hr. On the other hand, expression of iNOS was observed in the dermis of mouse ear tissues exposed to HN2. The iNOS was found within infiltrating immune cells in both the HN2 treated side (Fig. 6, Panel E), as well as the contralateral side of the dermis (untreated side, Fig. 6, Panel I). Ear tissues treated three times with EB-1 at a test dose of 0.250 mg/ear at 15 min, 4 hr and 8 hr after HN2 treatment (Fig. 6, Panels F and J) showed decreased tissue infiltration of iNOS positive (iNOS+) immune cells compared to HN2-exposed ear tissues treated thrice with DMSO (Fig. 6, compare Panels E and I to Panels F and J). HN2-exposed ear tissues treated thrice with HC (0.031 mg/ear) also showed less HN2-induced infiltration of iNOS + immune cells in the dermis (Fig. 6, compare Panels G and K to Panels E and I). Lastly, compared to HN2 exposed ears treated thrice with DMSO, the tissue expression of iNOS + immune cells observed in HN2 exposed ear tissues treated thrice with the combination of HC (0.031 mg/ear) + EB-1 (0.250 mg/ear) was dramatically reduced (Fig. 6, compare Panel H to Panels E and I). iNOS + cells were counted in all tissue sections and found to decrease in order of magnitude in the following pattern, HN2-exposed tissues > HN2-exposed tissues treated with EB-1 or HC alone or in combination (Supplementary Fig. 2, Panel C). In other words, the effect of the combination of HC + EB-1 on the quantity of iNOS + cells reduced those cells in HN2-treated tissues but was not stronger in this regard than either agent administered alone.

Summary of results

An overall summary of the findings for the 24 hr study can be found in Table 3. It is worthy to note that the effect of the combination of HC and EB-1 completely suppressed vesication by HN2 and exerted an additive effect with regard to the reduction of HN2-induced tissue edema, suggesting that HC and EB-1 are likely to be acting on distinct targets to reduce HN2 induced cutaneous damage (Table 4).

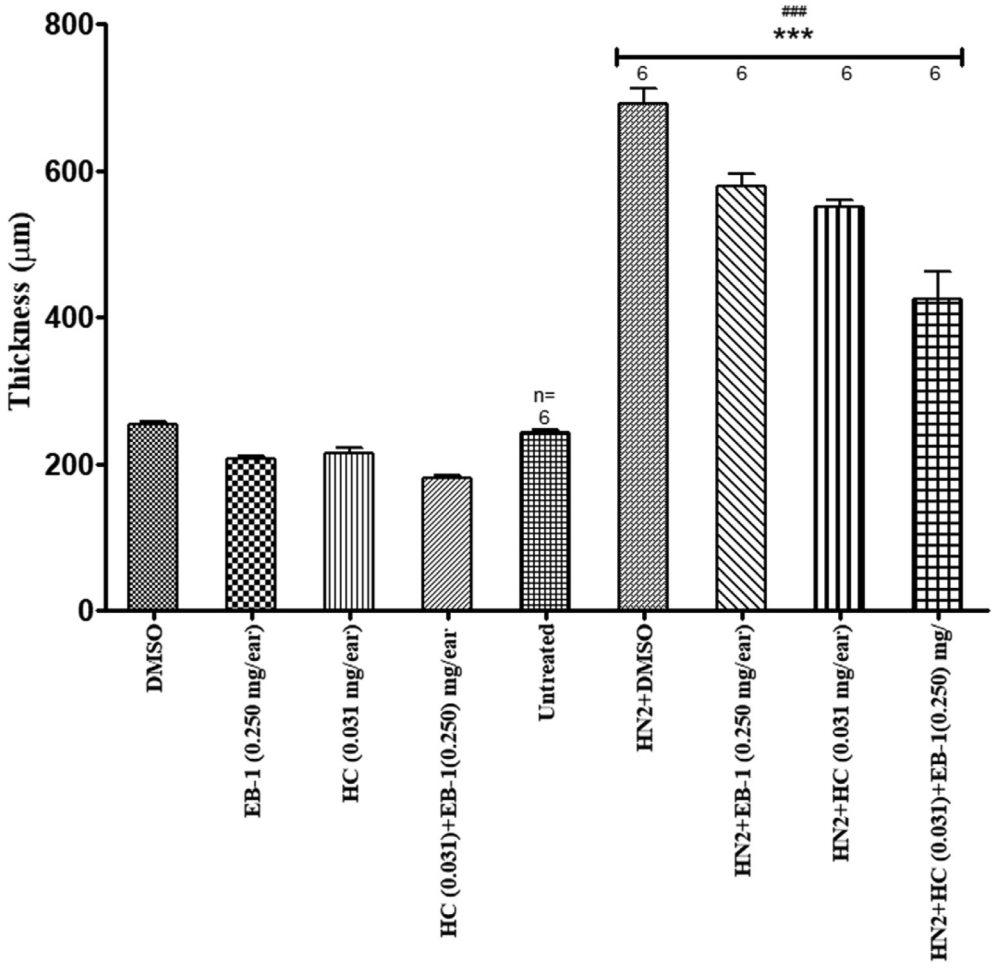

Fig. 3. Morphometric thickness of ear punches obtained from male Swiss Webster mice 24 h after topical exposure to HN2 (0.5 μmol/ear). Various treatment agents were applied at 15 min, 4 h and 8 h post HN2 exposure. Control ears were treated similarly, but without HN2 exposure. Untreated ear tissues were obtained from naive mice. Differences were statistically significant between DMSO group and HN2 treated groups at ***p < 0.001; and between the HN2 + DMSO group and the HN2 + EB-1 (0.250 mg/ear), HN2 + HC (0.031) and HN2 + HC (0.031) + EB-1 (0.250) groups at ###p < 0.001. Bars represent the mean ± SEM of the indicated number (n) of samples.

Table 2
Vesication/epithelial detachment in ear punches obtained from the male Swiss Webster mice at 24 h after topical exposure to 0.500 μmol/ear HN2 followed by DMSO, EB-1, HC and HC + EB-1 treatments[a].

Treatment (mg/ear)	Incidence of vesication (%)	n
Untreated	0	6
DMSO	0	6
HN2 + DMSO	100	6
HN2 + EB-1 (0.125 mg/ear)	0.0	6
HN2 + EB-1 (0.187 mg/ear)	16.7	6
HN2 + EB-1 (0.250 mg/ear)	16.7	6
HN2 + EB-1 (0.375 mg/ear)	33.3	6
HN2 + EB-1 (0.500 mg/ear)	50.0	6
HN2 + HC (0.016 mg/ear)	33.3	6
HN2 + HC (0.023 mg/ear)	33.3	6
HN2 + HC (0.031 mg/ear)	33.3	6
HN2 + HC (0.047 mg/ear)	16.7	6
HN2 + HC (0.063 mg/ear)	0.0	6
HN2 + HC (0.125 mg/ear)	0.0	6
HN2 + HC (0.250 mg/ear)	33.3	9
HN2 + HC (0.031) + EB-1 (0.250) mg/ear	0.0	6

[a]Vesication scoring of H&E stained sections obtained from right ears treated with vehicle (DMSO) or specific doses of EB-1, HC or HC + EB-1 after HN2 exposure. Data represents the incidence of vesication in the indicated number (n) of samples.

Discussion

Mechlorethamine (HN2) is an effective anticancer drug. However, serious adverse effects are associated with it, especially painful extravasation reactions (EVRs) which include tissue edema, inflammation and blistering (vesication) due to dermal-epidermal junction (DEJ) disruption (Ener et al., 2004). EVRs are reported to occur in up to 6% of patients receiving intravenous cancer chemotherapy (Bertelli, 1995; Cassagnol and McBride, 2009). Indeed, HN2 exhibits a very complex mechanism of toxicity that involves many macromolecules and cell signaling pathways (Korkmaz et al., 2006; McManus and Huebner, 2005; Tewari-Singh and Agarwal, 2016). Our group previously reported that HN2 triggers numerous responses in cultured skin cells *in vitro*, including damaged nuclear DNA, increased lipid peroxidation and apoptosis via activation of caspases 3 and 9 (Lulla et al., 2013; Pino and Billack, 2008; Pino et al, 2014), and that the organoselenium compound ebselen (EB-1) reduces the toxicity of both HN2 (Hardej and Billack, 2007; Lulla et al., 2014) and the sulfur half mustard CEES *in vitro* (Pino et al., 2013).

Despite our *in vitro* advances in understanding HN2 toxicity, there is an urgent need to investigate cutaneous responses to HN2 *in vivo* so as to more fully understand the mechanisms used by HN2 to achieve DEJ disruption. At least one of the cutaneous responses to HN2 observed in mouse skin involves increased tissue expression of MMP-

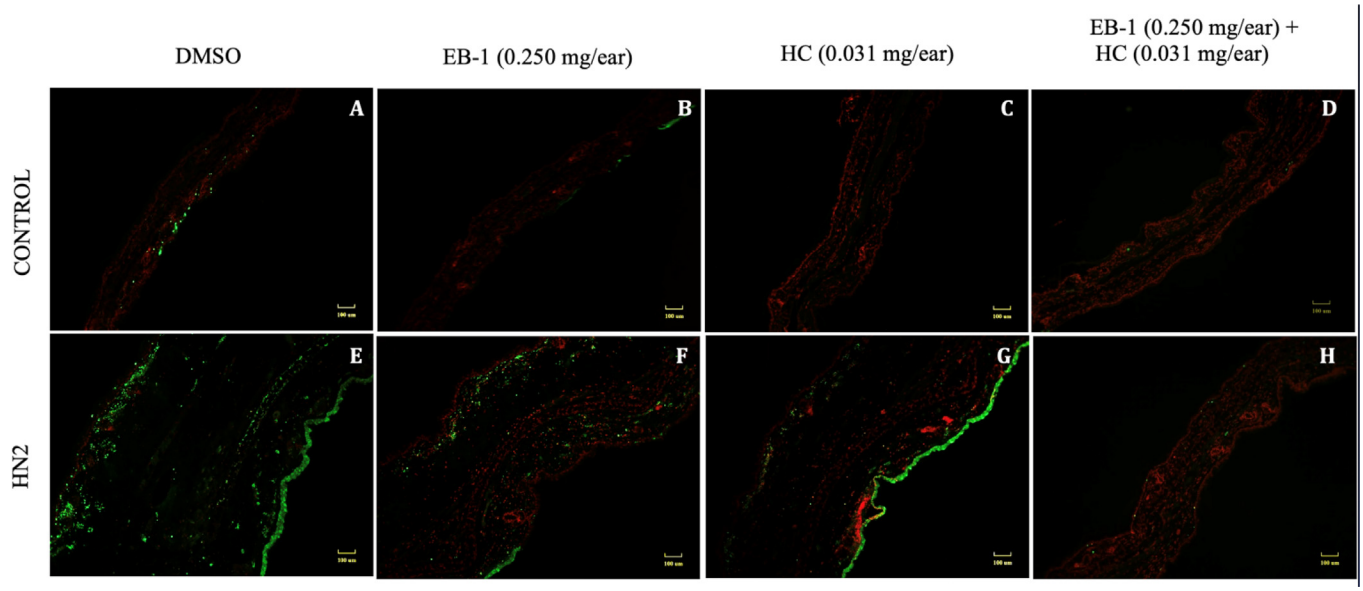

Fig. 4. Representative fluorescent TUNEL micrographs of ear tissue sections obtained from male Swiss Webster mice. TUNEL micrographs were obtained using samples isolated 24 h after topical administration of (A) vehicle (DMSO), (B) EB-1 (0.250), (C) HC (0.031), and (D) HC (0.031) + EB-1 (0.250) mg/ear at 15 min, 4 h and 8 h; and with 0.500 μmol/ear HN2 followed by three treatments (3X) of (E) vehicle (DMSO), (F) EB-1 (0.250), (G) HC (0.031) and (H) HC (0.031) + EB-1 (0.250) mg/ear at 15 min, 4 h and 8 h. Magnification used was 100×. Bars on the micrograph represent a length of 100 μm.

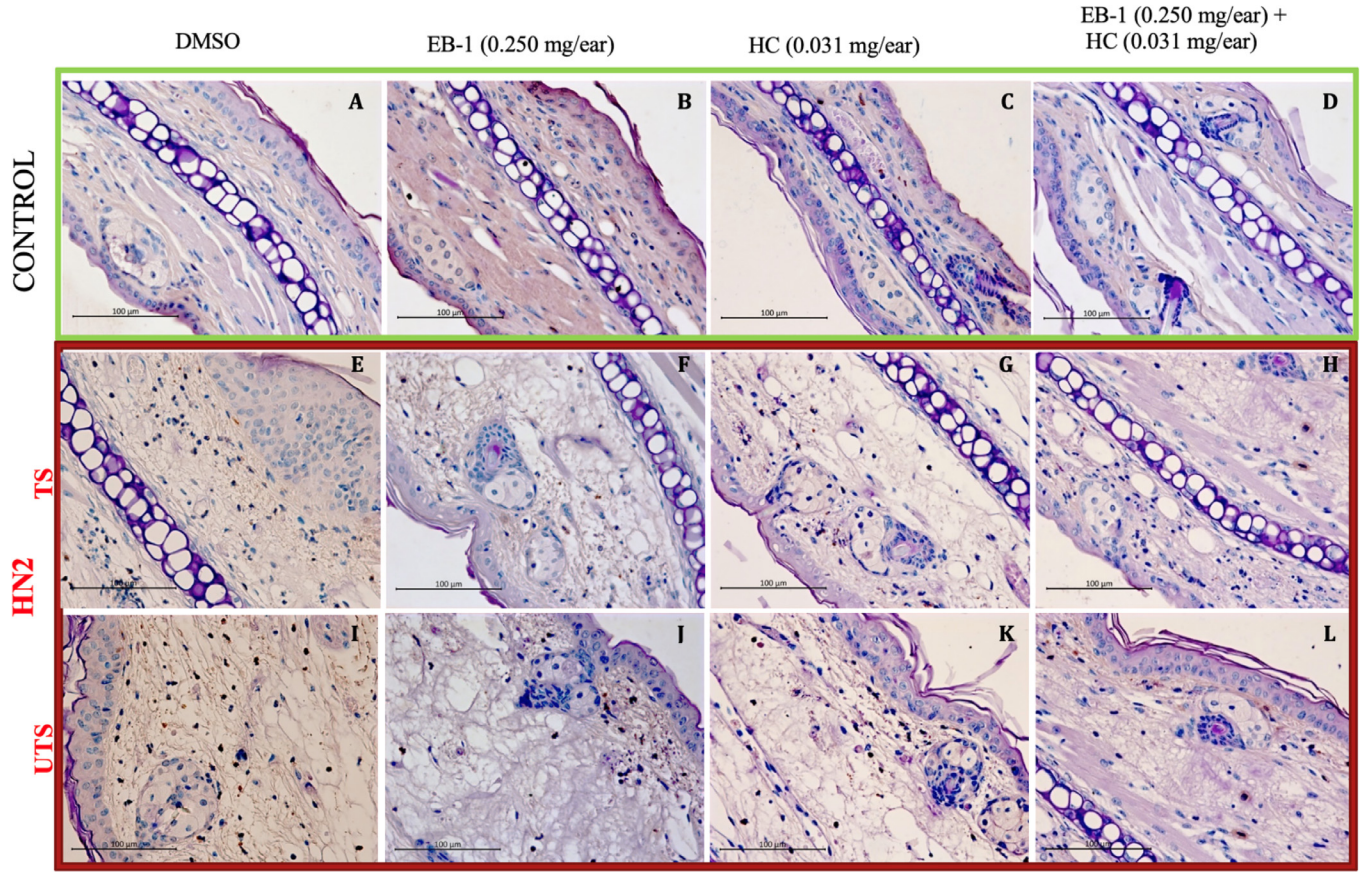

Fig. 5. Representative light micrographs of IHC staining for MMP-9 of ear tissue sections obtained from male Swiss Webster mice Light micrographs showing IHC for MMP-9 were obtained using samples isolated 24 h after topical administration of, (A) vehicle (DMSO), (B) EB-1 (0.250), (C) HC (0.031) and (D) HC (0.031) + EB-1 (0.250) mg/ear at 15 min, 4 h and 8 h; and with 0.500 μmol/ear HN2 followed by three treatments (3X) of (E and I) vehicle (DMSO), (F and J) EB-1 (0.250), (G and K) HC (0.031) and (H and L) HC (0.031) + EB-1 (0.250) mg/ear at 15 min, 4 h and 8 h. Total magnification used was 400×. Bars on the micrographs represent a length of 100 μm. TS: Treated side; UTS: Untreated side.

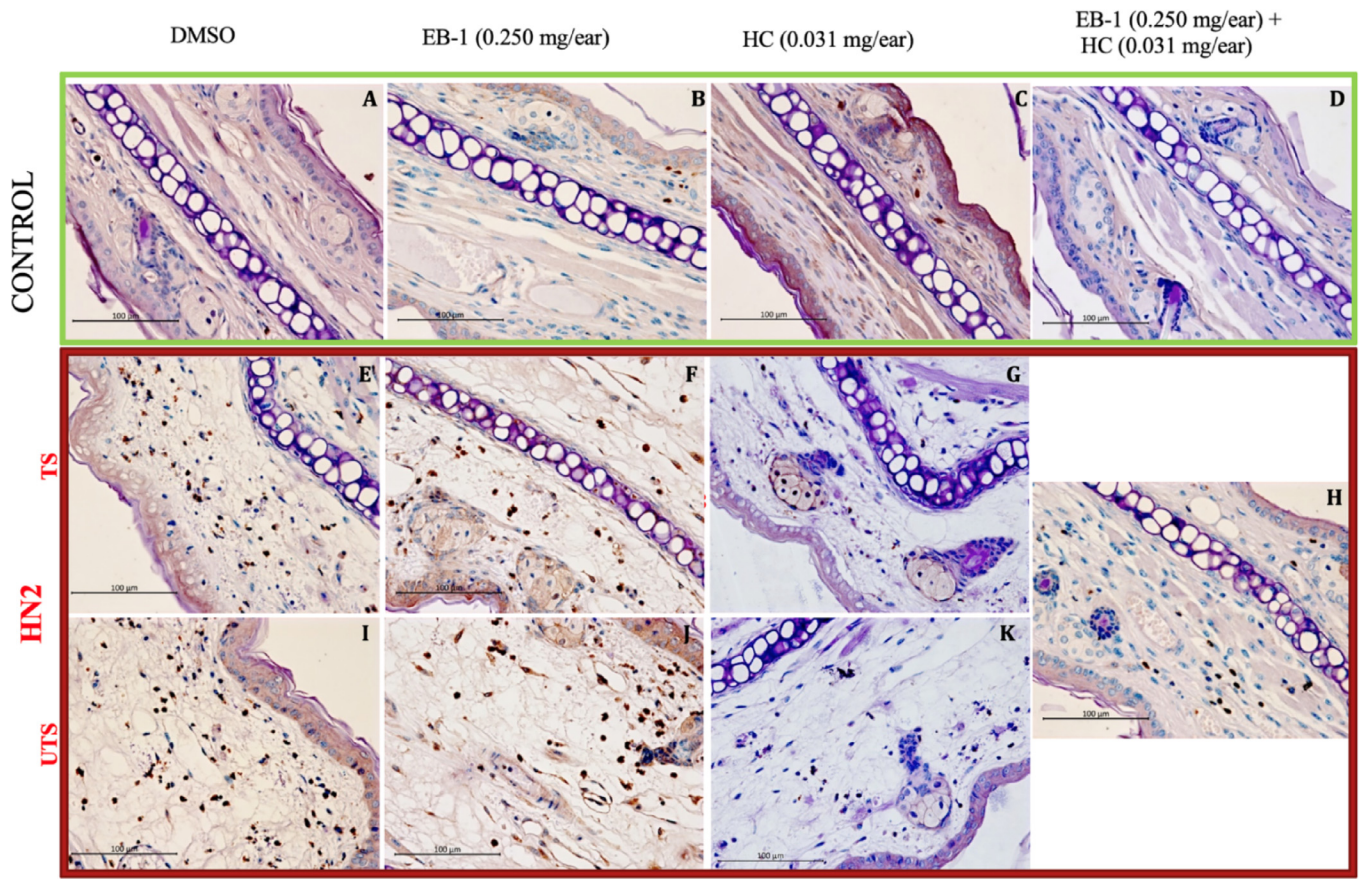

Fig. 6. Representative light micrographs of IHC staining for iNOS of ear tissue sections obtained from male Swiss Webster mice Light micrographs showing IHC for iNOS were obtained using samples isolated 24 h after topical administration of (A) vehicle (DMSO), (B) EB-1 (0.250), (C) HC (0.031) and (D) HC (0.031) + EB-1 (0.250) mg/ear at 15 min, 4 h and 8 h; and with 0.500 μmol/ear HN2 followed by three treatments (3X) of (E and I) vehicle (DMSO), (F and J) EB-1 (0.250), (G and K) HC (0.031) and (H) HC (0.031) + EB-1 (0.250) mg/ear at 15 min, 4 h and 8 h. Total magnification used was 400×. Bars on the micrographs represent a length of 100 μm. TS: Treated side; UTS: Untreated side.

Table 3
Summary of the HN2 countermeasure potential of the HC + EB-1 combination.

Parameter	HC + EB-1 effect on HN2 injury
H & E Micrographs	↓↓
Tissue Morphometry	↓↓
Vesication Score	↓↓
TUNEL Assay Micrographs	↓↓
MMP9 IHC Micrographs	↓↓
iNOS IHC Micrographs	↓↓

Table 4
Percentage reduction of HN2 cutaneous toxicity by the HC + EB-1 combination

24 h treatment	% Reduction in morphometry vs. HN2 + DMSO	% Incidence of vesication
DMSO vehicle	–	100.0
EB-1 (0.250 mg/ear)	16.2	16.7
HC (0.031 mg/ear)	20.4	33.3
HC (0.031) + EB-1 (0.250) mg/ear	38.7	0.0

9, a matrix metalloproteinase that directly degrades extracellular matrix (ECM) proteins and activates cytokines and chemokines to regulate tissue remodeling in response to chemical injury. Our lab (Cuffari et al., 2018; Tumu et al., 2020) and others (Kumar et al., 2015; Wormser et al., 2002) have found that HN2 increases the expression of MMP-9 in mouse skin. In addition, we (Tumu et al., 2020) and others (Biyashev et al., 2020; Composto et al., 2016; Kumar et al., 2015) have observed that HN2 increases the tissue expression of inducible nitric oxide synthase (iNOS) in mouse skin, which, in turn, is important for generating cytotoxic levels of nitric oxide in the skin (Composto et al., 2016; Nguyen and Soulika, 2019). Moreover, we have also reported that a significant increase in TUNEL positive cells occurs in mouse ear skin treated with HN2 (Tumu et al., 2020). Therefore, for the present study, we have implemented an operational definition of dermatotoxicity to include tissue responses to HN2 that are consistent with increases in the morphometric thickness of H&E-stained ear sections; histopathologic observations including increased tissue edema and vesication, and observation of increased tissue expression of MMP-9 and iNOS. In this working definition, a drug that reduces the dermatoxocity of HN2 will therefore be one that reduces each of these tissue responses.

The primary aim of the present work was therefore to investigate and more fully characterize the pharmacologic effects of HC and EB-1, both alone and in combination, on the cutaneous responses to the DEJ disruptor HN2 using a mouse model; namely, the MEVM. The MEVM was originally used to study sulfur mustard toxicity (Casillas et al., 1997; Casillas et al., 2000; Dachir et al., 2002; Shakarjian et al., 2006; Smith et al., 1997). Several laboratories, including ours, have successfully adapted the model to study the toxicity of HN2 or other DEJ disruptors (e.g., 2-chloroethyl ethyl sulfide) *in vivo* (Achanta et al., 2018; Cuffari et al., 2018; Lulla et al., 2014; Tumu et al., 2020; Young et al., 2012). While both EB-1 (Lulla et al., 2014)

and HC (Casillas et al., 2000; Dachir et al., 2002) have been previously studied in the MEVM, the primary endpoints measured in those studies were ear edema and histopathology. No other investigation of the effects of EB-1 or HC, to our knowledge, on HN2-induced vesication, TUNEL activity, iNOS or MMP-9 expression in mouse skin has been reported to date.

Therefore, in the present study, these two test compounds were each investigated separately and in combination for their ability to reduce the dermatoxicity of HN2. EB-1 is an anti-inflammatory antioxidant compound that is under investigation for reperfusion injury (Aras et al., 2014), stroke (Yamaguchi et al., 1998), hearing loss (Kil et al., 2021) and bipolar disorder (Singh et al., 2013). Previous research in our lab has shown the edema-reducing and anti-vesicant (Lulla et al., 2014), anti-bacterial (Chan et al., 2007), anti-fungal (Billack et al., 2010; Orie et al., 2017) and cyto-protective (Hardej and Billack, 2007; Pino and Billack, 2008; Pino et al., 2013) properties of EB-1. The second compound tested here was HC, a corticosteroid with immunosuppressant activity (Issekutz, 1983, Coutinho and Chapman, 2011), is used topically in the treatment of various inflammatory skin conditions such as allergic rashes, eczema and psoriasis. It should be noted that an important hypothesis tested in this work was that combination treatment with HC and EB-1 is beneficial in treating DEJ disruption and cutaneous injury by HN2. The straightforward rationale behind the combination approach is that since multiple cytotoxic pathways are activated by HN2, two drugs acting via different pathways should be better than one for arresting or reducing tissue injury and, to our knowledge, ours is the first study to investigate this specific combination for countermeasure activity against DEJ disruption by HN2. While several combination doses were tested and observed to be effective (Supplemental Fig. 1c), the most effective dose found to reduce tissue edema was HC (0.031 mg/ear) and EB-1 (0.250 mg/ear). Thus, this combination was further assessed for efficacy by H&E, IHC for MMP-9 and iNOS and TUNEL assay. Indeed, H&E staining revealed that mouse ears treated HN2 followed by the combination of HC (0.031 mg/ear) and EB-1 (0.250 mg/ear) showed a marked similarity to untreated vehicle control (DMSO) ears. This combination also reduced MMP-9, iNOS and TUNEL staining in HN2-treated tissues.

Again, for the combination of HC and EB-1, each component was selected from a different pharmacologic class, with EB-1 being an organoselenium compound and HC being a glucocorticoid. Thus, while it remains to be confirmed, it is unlikely that pharmacologic redundancy was encountered in this work, as there potentially could be if two members of the same pharmacologic class had been selected as a combination treatment for testing in the MEVM (e.g., dexamethasone and HC are both glucocorticoids and their use in combination could lead to excessive immunosuppression of the skin). It is also noteworthy that the combination reduced vesication incidence to 0% (none of the 6 mice showed vesication) and reduced morphometric thickness by 38.7%. The effects of the HC and EB-1 combination appear to be additive with regard to morphometric thickness (HC reduced it 20.4%, EB-1 reduced it by 16.2% and the combination reduced it 38.7%). It is important to recognize that the combination of HC + EB-1 completely abrogated vesication which points to a possible role for this combination in the treatment of nitrogen mustard-associated EVRs. Moreover, it will be worth investigating the extent to which this combination can ameliorate sulfur mustard-induced tissue blistering.

It should be noted that when EB-1 was administered as monotherapy, the data showed an overall superior protection against HN2-induced vesication at lower test doses of EB-1 (0.125, 0.187 and 0.250 mg/ear) than at the higher test doses (0.375 and 0.500 mg/ear). To be specific, ears exposed to HN2 and then treated with 0.125 mg/ear of EB-1 showed 0% vesication compared to 50% vesication when exposed to HN2 and treated with 0.500 mg/ear of EB-1. The finding that lower test doses of EB-1 are more effective than higher ones at reducing dermatoxicity was unexpected; however, other studies have also reported a worsening of protection upon increasing doses of intervention. For example, dexamethasone (once a day) offered more protection in terms of reducing sulfur mustard-induced ear edema, than applying the same dose of dexamethasone thrice a day (Dachir et al., 2002). In a study published by Wormser and colleagues, anti TNF-α antibodies reduced sulfur mustard induced ear swelling to a larger degree at 1.0 mcg/mouse than at 2.0 mcg/mouse (Wormser et al., 2005). Brodsky and colleagues have also observed that low and high doses of an anti-inflammatory peptide showed less protection against SM when compared to moderate doses of the peptide (Brodsky et al., 2008). More work will be needed to better understand this effect.

There are at least two limitations to the study at hand that should be noted. First, as discussed previously (Lulla et al., 2014; Tumu et al., 2020), it remains to be determined as to whether the results obtained with the MEVM can be generalized to human skin. Compared to mouse skin, human skin has a thicker and multi-layered epidermis, sweat glands, sebaceous glands and melanin. In contrast, the ear tissues of the Swiss Webster mice used here have what appears to be a single layered epidermis and sebaceous glands, and lack both sweat glands and melanin. More work is therefore required to determine the extent to which these observations will apply to human skin responses to DEJ disruption by HN2. The second limitation is the vehicle we have chosen, since DMSO has a slight anti-inflammatory property (Hollebeeck et al., 2011). While the ears treated here with DMSO did not exhibit statistically significant changes in morphometric thickness analysis, there may be subtler molecular effects of this vehicle at time points earlier than 24 hr (Cuffari et al., 2018), thereby warranting further study.

It is also worth pointing out that the increase in TUNEL fluorescence induced by HN2 and its inhibition by EB-1, HC and the combination thereof may not necessarily implicate only apoptosis as part of the mechanism of HN2 dermatotoxicity. TUNEL staining may also be used to detect DNA damage associated with non-apoptotic events, such as necrotic cell death induced by exposure to toxic compounds and other insults (Ansari et al., 1993), and TUNEL staining has also been reported to stain cells undergoing active DNA repair (Kanoh et al., 1999). It is clear from our study that EB-1 reduces HN2-driven TUNEL fluorescence by itself or in combination with HC. More work will be required to determine which pathway is being inhibited by EB-1 and whether the combination of HC and EB-1 is reducing apoptosis or not, and, if so, whether that inhibition is directly due to the drugs or is instead an indirect effect manifesting itself but resulting from the inhibition of some other target in the tissue by these drugs. In fact, the current study is unable to definitively point to iNOS and MMP-9 inhibition in the tissue as direct effects of drug treatment or downstream effects caused by the inhibition of a yet-to-be identified molecule or signaling pathway in the tissue. Nonetheless, the combination treatment certainly was effective in reducing DEJ disruption by HN2, which lays the groundwork for future studies in human skin models.

CRediT authorship contribution statement

Hemanta C Rao Tumu: Conceptualization, Methodology, Formal analysis, Writing – original draft, Writing – review & editing. **Benedette J. Cuffari:** Methodology, Formal analysis, Writing – review & editing. **Blase Billack:** Conceptualization, Methodology, Formal analysis, Writing – original draft, Writing – review & editing, Supervision, Funding acquisition.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.crtox.2021.10.002.

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