Experimental and Molecular Pathology 100 (2016) 522–531

Contents lists available at ScienceDirect

Experimental and Molecular Pathology

journal homepage: www.elsevier.com/locate/yexmp

Mitigation of nitrogen mustard mediated skin injury by a novel indomethacin bifunctional prodrug

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A R T I C L E   I N F O

Article history:
Received 11 March 2016
and in revised form 26 April 2016
Accepted 13 May 2016
Available online 14 May 2016

Keywords:
Vesicants
Sulfur mustard
Anti-cholinergic
Epidermis
Countermeasures

A B S T R A C T

Nitrogen mustard (NM) is a bifunctional alkylating agent that is highly reactive in the skin causing extensive tissue damage and blistering. In the present studies, a modified cutaneous murine patch model was developed to characterize NM-induced injury and to evaluate the efficacy of an indomethacin pro-drug in mitigating toxicity. NM (20 μmol) or vehicle control was applied onto 6 mm glass microfiber filters affixed to the shaved dorsal skin of CD-1 mice for 6 min. This resulted in absorption of approximately 4 μmol of NM. NM caused localized skin damage within 1 d, progressing to an eschar within 2–3 d, followed by wound healing after 4–5 d. NM-induced injury was associated with increases in skin thickness, inflammatory cell infiltration, reduced numbers of sebocytes, basal keratinocyte double stranded DNA breaks, as measured by phospho-histone 2A.X expression, mast cell degranulation and increases in inducible nitric oxide synthase (iNOS) and cyclooxygenase-2 (COX-2). Wound healing was characterized by epidermal hyperplasia and marked increases in basal cells expressing proliferating cell nuclear antigen. A novel indomethacin-anticholinergic prodrug (4338) designed to target cyclooxygenases and acetylcholinesterase (AChE), was found to markedly suppress NM toxicity, decreasing wound thickness and eschar formation. The prodrug also inhibited mast cell degranulation, suppressed keratinocyte expression of iNOS and COX-2, as well as markers of epidermal proliferation. These findings indicate that a novel bifunctional pro-drug is effective in limiting NM mediated dermal injury. Moreover, our newly developed cutaneous patch model is a sensitive and reproducible method to assess the mechanism of action of countermeasures.

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1. Introduction

Sulfur mustard (bis(2-chloroethyl) sulfide) and nitrogen mustard (NM, bis(2-chloroethyl) methylamine, mechlorethamine) are highly toxic bifunctional alkylating agents causing epidermal and dermal damage (Shakarjian et al., 2010). In humans, depending on the dose and duration of exposure, mustards can cause acute injury, inflammation, the formation of ulcerative wounds, and blistering (Arck and Paus, 2006; Ghabili et al., 2010; Vogt et al., 1984). Generally similar responses are observed in rodent models of cutaneous exposure to sulfur mustard or NM (Tewari-Singh et al., 2013).

Key to elucidating the mechanism of action of mustards and testing potential countermeasures is the ability to generate reproducible injury in localized areas of the skin in experimental animal models. Since sulfur mustard is both lipophilic and volatile, dermal exposure can be localized using vapor cup models (Anderson et al., 2002; Joseph et al., 2011; Mershon et al., 1990). In contrast, NM is hydrophilic; thus, direct application in solvents results in spreading over a relatively large area of skin. This makes quantification of tissue damage difficult to assess (Tewari-Singh et al., 2013; Tewari-Singh et al., 2014; Vieille-Petit et al., 2015). To address this problem, cutaneous patch models have been developed. In these models, which have been used in both humans and animals, test chemicals are suspended in a matrix, which is attached to a designated area of the skin with occlusive or semi-occlusive tape (Buehler, 1994; Farage et al., 2011; Fischer and Kihlman, 1989; Nicholson and Willis, 1999). A similar model has been developed in guinea pigs to analyze hypersensitivity reactions (Buehler, 1994). Patch models have also been used to evaluate treatments for chemical burns, and as a method for delivering hydrophilic substances to the

Abbreviations: (AChE), acetylcholinesterase; (COX), cyclooxygenases; (D/E), dermal/epidermal; (H&E), hematoxylin & eosin; (NM), nitrogen mustard; (PCNA), proliferating cell nuclear antigen; (phospho-H2A.X), phosphorylated histone H2A.X; (PGE_2), prostaglandin E2; (SM), sulfur mustard.

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http://dx.doi.org/10.1016/j.yexmp.2016.05.008
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skin (Hojer et al., 2002; Hulten et al., 2004). Although many patch models can be extrapolated to human skin, there are limitations including differences between humans and animals in skin permeability of test agents, as well as their ability to induce irritation reactions (Chew and Maibach, 2003; Simon and Maibach, 1998).

In the present studies, a modified semi-occlusive patch test model employing a glass microfiber filter delivery system was developed for cutaneous NM delivery in mice; our goal was to characterize skin injury and wound healing, and to assess the efficacy of a novel bifunctional anti-inflammatory prodrug, 4338, as a potential countermeasure. The advantage of microfiber patch dosing is that it provides an effective method of applying NM over defined areas of the skin, making it easier to quantify tissue damage. The prodrug (4338) was designed to target cyclooxygenases (COX), enzymes that generate proinflammatory eicosanoids, and also to target acetylcholinesterase, an enzyme mediating hydrolysis of acetylcholine (see Fig. 1 for structure). The advantage of the prodrug is that it provides a simple dosage form for two drug targets in the skin and a facilitated mechanism for onsite controlled release of the individual therapeutic components. This provides an opportunity for therapeutic properties on the skin that exceed those of the component parts. In earlier studies we showed that 4338 was effective in reducing sulfur mustard-induced edema, as well as degradation of the dermal-epidermal basement membrane; it also reduced expression of COX-2 and promoted wound re-epithelialization (Chang et al., 2014). The present studies demonstrate that our modified cutaneous patch test model is highly reproducible and can be used to effectively evaluate candidate therapeutics with the potential to mitigate vesicant-induced skin injury.

2. Materials and methods

2.1. Chemicals and reagents

Polyethylene glycol 400 and lanolin (C10–30 cholesterol/lano-sterol esters) were from Croda Inc., Edison, NJ. Unless otherwise indicated NM (mechlorethamine HCl) and all other chemicals were purchased from Sigma-Aldrich (St. Louis, MO). 4338 was synthesized as previously described (Young et al., 2010).

2.2. Animals and treatments

Female CD-1 mice (8 weeks; Charles River Laboratories, Wilmington, MA) were housed in filter-top microisolation cages and maintained on food and water ad libitum. Mice received humane care in compliance with the Rutgers University guidelines, as outlined in the *Guide for the Care and Use of Laboratory Animals* published by the National Institutes of Health. Mice were anesthetized by intraperitoneal injection of ketamine (80 mg/kg) and xylazine (12 mg/kg), and randomly assigned to treatment groups. The dorsal skin of the mice was shaved and then cleaned with deionized water. Six mm glass microfiber filter discs (GE Healthcare, Buckinghampshire, UK) were placed on the lumbar region of the shaved skin equal distant from the spine, and adhered with 10 μl of acetone (Fig. 2). Twenty μl of freshly prepared 1 M NM (20 μmol) in 20% deionized water/80% acetone, v/v, was then applied to the filters and immediately covered with PARAFILM® (Pechiney, Menasha, WI). Control mice received the solvent without NM. After 6 min, the filter discs were removed. In some experiments, mice were treated topically with 4338 four times per day beginning 1 h after NM. For these experiments, 20 μl of 4338 (1% in a mixture of 49.5% lanolin and 49.5% PEG400) or vehicle control was applied directly to the skin. Mice were euthanized 1–5 days post NM and 12 mm full thickness biopies of the exposure site and surrounding tissue immediately collected using a skin punch (Acuderm Inc., Ft. Lauderdale, FL). Punch biopsies were trimmed and stored in ice cold phosphate buffered saline (PBS) containing 3% paraformaldehyde/2% sucrose. The tissue was embedded in paraffin and 6 μm sections prepared and stained with hematoxylin and eosin (HE) or Gomori's trichrome containing methyl (aniline) blue, for analysis of collagen I/III (Goode Histolabs, New Brunswick, NJ). Hematoxylin and eosin stained tissue was scanned using a VS120-L100 Olympus virtual slide microscope (Waltham, MA). In some experiments, histological sections were stained with toluidine blue O (Sigma Chemical, St. Louis MO) to visualize metachromatic/basophilic granules in mast cells. Extrusion of basophilic toluidine blue stained granules was evidence of mast cell degranulation (Joseph et al., 2011). To quantify wound thickness, measurements were performed on tissue sections using the OlyVIA 2.7 viewer software (Olympus). Skin sections were divided into 4 equal parts and measurements taken perpendicular from wound edge through the dermis, to the top surface of the hypodermis. Data were analyzed using a two way ANOVA and were expressed as mean $\pm$ S.E. ($n = 6$). A p value ≤0.05 was considered significant.

To estimate absorption of NM from the filter discs, NM was applied to 6 weighed control discs and allowed to dry. The filters were then weighed again to determine filter bound NM. Twelve weighed discs treated with NM that had been applied to mice (treatment discs) were removed and dried. These filters were also weighed again to

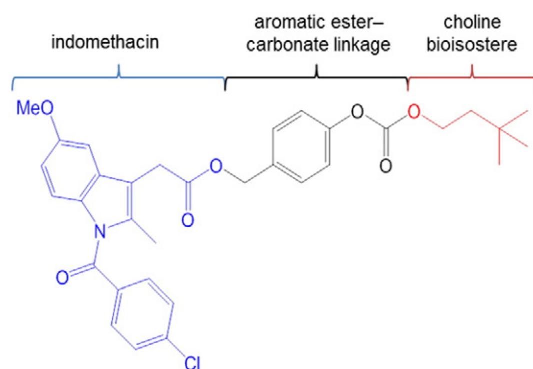

Fig. 1. Chemical structure of 4338. 4338 consists of an anti-inflammatory moiety, indomethacin, and a choline bioisostere 3.3-dimethyl-1-butanol, linked via an aromatic ester–carbonate.

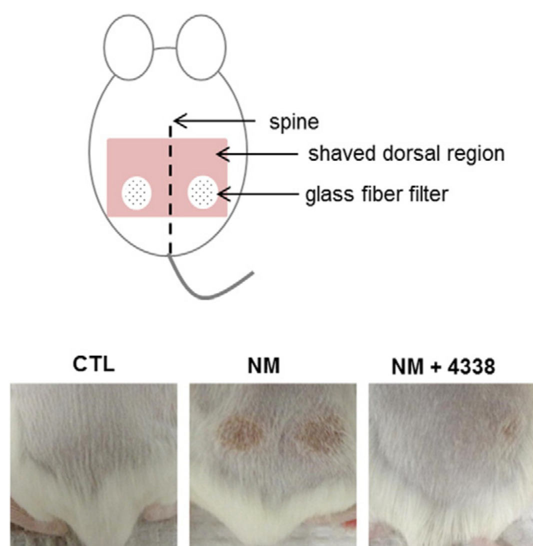

Fig. 2. Modified dorsal skin patch model. The dorsal skin of CD-1 mice was shaved and 6 mm glass fiber filter discs placed on the lumbar region of the skin equal distant from the spine (upper panel). Twenty microliters of a 1 M solution of NM in 20% deionized water/80% acetone (v/v) or vehicle control was applied to the filters which were then covered with PARAFILM®. After 6 min, the filter discs were removed and the skin analyzed for tissue damage 1–5 days post exposure. Mice were treated with 4338 four times per day beginning 1 h post NM exposure. The lower panel shows the skin from control (CTL), NM and NM + 4338 treated skin 3 days post NM.

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