Published in final edited form as:

Toxicol Appl Pharmacol. 2014 October 15; 280(2): 236–244. doi:10.1016/j.taap.2014.07.016.

Therapeutic Potential of a Non-Steroidal Bifunctional Anti-Inflammatory and Anti-Cholinergic Agent against Skin Injury Induced by Sulfur Mustard

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Abstract

Sulfur mustard (bis(2-chloroethyl) sulfide, SM) is a highly reactive bifunctional alkylating agent inducing edema, inflammation, and the formation of fluid-filled blisters in the skin. Medical countermeasures against SM-induced cutaneous injury have yet to be established. In the present studies, we tested a novel, bifunctional anti-inflammatory prodrug (NDH 4338) designed to target cyclooxygenase 2 (COX2), an enzyme that generates inflammatory eicosanoids, and acetylcholinesterase, an enzyme mediating activation of cholinergic inflammatory pathways in a model of SM-induced skin injury. Adult SKH-1 hairless male mice were exposed to SM using a dorsal skin vapor cup model. NDH 4338 was applied topically to the skin 24, 48, and 72 hr post-SM exposure. After 96 hr, SM was found to induce skin injury characterized by edema, epidermal hyperplasia, loss of the differentiation marker, keratin 10 (K10), upregulation of the skin wound marker keratin 6 (K6), disruption of the basement membrane anchoring protein laminin 322, and increased expression of epidermal COX2. NDH 4338 post-treatment reduced SM-induced dermal edema and enhanced skin re-epithelialization. This was associated with a reduction in COX2

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Conflict of interest statement

The authors declare that there are no conflicts of interest.

expression, increased K10 expression in the suprabasal epidermis, and reduced expression of K6. NDH 4338 also restored basement membrane integrity, as evidenced by continuous expression of laminin 332 at the dermalepidermal junction. Taken together, these data indicate that a bifunctional anti-inflammatory prodrug stimulates repair of SM induced skin injury and may be useful as a medical countermeasure.

Keywords

skin; sulfur mustard; Non-steroid bifunctional anti-inflammatory and anticholinergic agent; wound repair

INTRODUCTION

Sulfur mustard (bis(2-chloroethyl) sulfide, SM) is a highly toxic vesicant that can rapidly penetrate human skin and cause extensive chemical burns (Vogt *et al.*, 1984; Shakarjian *et al.*, 2010). As a reactive bifunctional alkylating agent, SM can form mono-adducts and cross-links with many nucleophilic components in cells including proteins, lipids, and DNA. This can result in prolonged inflammation and delayed wound repair (Papirmeister *et al.*, 1985; Rowell *et al.*, 2009). SM can also alkylate basement membrane components in the skin which disrupts the dermal-epidermal junction (DEJ), causing detachment of basal keratinocytes from the basement membrane (Monteiro-Riviere and Inman, 1997; Petrali and Oglesby-Megee, 1997; Monteiro-Riviere *et al.*, 1999). Both apoptosis and necrosis of keratinocytes are thought to contribute to SM-induced skin vesication (Petrali and Oglesby-Megee, 1997; Rosenthal *et al.*, 1998; Ruff and Dillman, 2007). Inflammatory cells and mediators are known to play a key role in the pathogenic response to SM (Casillas *et al.*, 2000; Sabourin *et al.*, 2000; Kehe *et al.*, 2009). Thus, the development of anti-inflammatory drugs with multiple mechanisms of action which target inflammatory signaling pathways represents a novel strategy for limiting SM-induced injury and promoting wound repair.

Non-steroidal anti-inflammatory drugs (NSAIDs) are the most commonly used agents to suppress inflammation. One important NSAID target is cyclooxygenase (COX), a family of enzymes which catalyze the synthesis of eicosanoids (Katori and Majima, 2000). Previous studies have shown that COX2 knockout mice are protected from SM-induced acute skin injury and that COX2 inhibitors suppress SM toxicity, demonstrating the importance of this enzyme in SM-induced skin damage (Babin *et al.*, 2000; Casillas *et al.*, 2000; Wormser *et al.*, 2004). Indomethacin was chosen because it had, in a pre-treatment based study, been reported to display modest edema-suppression in rodents exposed to sulfur mustard (Casillas *et al.*, 2000). Also, in an earlier publication from our laboratory using a post-treatment assay, Indomethacin ranked better than Diclofenac, Ibuprofen, and S-Naproxen when incorporated into a prodrug, dually targeting inflammation and cholinergic dysfunction (Young *et al.*, 2012).

Evidence also suggests that pro-inflammatory molecules generated following injury in the skin are regulated via cholinergic anti-inflammatory pathways (Kurzen *et al.*, 2007). In this regard, cholinergic antagonists including acetylcholinesterase inhibitors (AChEI) have been reported to be effective in suppressing inflammation (Tracey, 2007; Reale *et al.*, 2009). The

effects of AChEI on skin inflammation and injury are unknown and were investigated in the present studies. We selected 3,3-dimethyl-1-butanol because it has been reported to be a weak ($K_i = 7.3$ mM), reversible, noncompetitive inhibitor of AChE and can be incorporated into esters as a bioisostere for choline and activate the cholinergic anti-inflammatory pathway (Cohen *et al.*, 1985). We have previously shown that although the 3,3-dimethylbutyl moiety does confer anticholinergic activity, the IC_{50} 's (as AChEIs) varied from 0.51 micromolar to >2000 micromolar depending on the particular NSAID to which the 3,3-dimethylbutyl piece is conjugated (Young *et al.*, 2010).

In these experiments, we utilized a prodrug consisting of an NSAID (indomethacin) and an AChEI (3, 3-dimethyl-1-butanol) (Young *et al.*, 2010). Indomethacin is tethered by ester and carbonate linkages to 3, 3-dimethyl-1-butanol, an anti-cholinergic choline bioisostere (Figure 1). This compound, referred to as NDH 4338, possesses anti-inflammatory activity and is an AChEI inhibitor (Young *et al.*, 2010; Young *et al.*, 2012). In previous studies using the mouse ear vesicant model we demonstrated that NDH 4338 mitigated skin inflammation induced by the half mustard vesicant, 2-chloroethyl ethyl sulfide (CEES) and was shown to reduce edema by over 90%, suggesting its use as a countermeasure against SM-induced skin injury (Young *et al.*, 2010; Young *et al.*, 2012). In the present studies we evaluated the efficacy of NDH 4338 against SM-induced skin injury using the hairless mouse skin model.

MATERIALS AND METHODS

Animals and treatments

Male SKH-1 hairless mice (Charles River Laboratories, Portage, MI), 5–8 weeks of age ($n = 6$ mice per group), were exposed to SM or sham control for 6 min using a vapor cup dorsal skin model as previously described (Joseph *et al.*, 2011). The SKH1 mice, used in the experiment, do have a normal coat of hair with normal appendages up to 10 days of age; after the first hair cycle, mature hair is lacking, although the appendages remain (Benavides *et al.*, 2009). The dorsal skin of the mice was treated with vehicle (dimethyl isosorbide (DMI)/ethyl oleate (Croda, Inc., Edison, NJ)) or vehicle formulated with 1% NDH 4338 at 24, 48 and 72 hr post SM exposure. Mice were sacrificed 96 hr after SM, punch biopsies of exposed areas collected, and divided into 2 sections. Skin tissue was either embedded in Tissue Tek optimum cutting temperature medium (OCT, Sakura Finetek USA, Torrance, CA) and flash frozen for histology, or directly flash frozen for protein analyses. SM exposures were performed on anesthetized mice and were carried out at Battelle Biomedical Research Center, West Jefferson, OH, USA. All animal protocols were approved by the Battelle Memorial Institute animal care and use committee. SM was obtained from Edgewood Chemical Biological Center and was chemically analyzed to be 99% pure. NDH 4338, was synthesized as previously reported (Young *et al.*, 2010).

Histology

Frozen sections (10 μ m) were fixed in Pen-Fix fixative (Thermo Scientific, PA), and stained with hematoxylin and eosin (H&E). Skin sections from three mice were evaluated for edema, necrosis and inflammation using an Olympus VS120 Virtual Scanning Microscopy System. To measure skin thickness, ten random sites from each skin section within the

wounded area were assessed. The distance from the stratum corneum to the dermal-epidermal junction (DEJ) was measured as the epidermal thickness; and the distance from the DEJ to the striated muscle was measured as the dermal thickness. The mean was calculated and analyzed for significance at $p < 0.05$ using the unpaired Student's t test.

Western blotting

Frozen skin biopsies were pulverized using a GenoGrinder as previously reported (Chang *et al.*, 2009). Tissue samples were lysed with RIPA buffer (50 mM Tris-HCl pH 7.4, 150 mM NaCl, 1 mM EDTA, 1% NP-40, 1% Na-deoxycholate and 0.1% SDS) and protein concentration determined using the BCA Protein Assay Reagent (Pierce Chemical, Rockford, IL). Pooled tissue lysates were analyzed on 7.5% sodium dodecyl sulfate-polyacrylamide gels and then transferred onto nitrocellulose membranes (Bio-Rad Laboratories, Life Sciences Group, Hercules, CA). The membranes were incubated with rabbit anti-COX2 polyclonal antibody (1:1,000, Abcam, Cambridge, MA) overnight at 4°C, or rabbit anti-GAPDH polyclonal antibody (1:20,000, Sigma-Aldrich, St. Louis, MO) for 1 h at room temperature, followed by goat antirabbit IgG horseradish peroxidase antibody (1:6,000–1:20,000, BioRad, Hercules, CA). Proteins were detected by chemiluminescence using the SuperSignal West Pico Chemiluminescence Substrate System (Pierce Chemical). GAPDH was used to ensure equal protein loading on the gels.

Immunofluorescence and confocal microscopy

Frozen sections (10 μ m) were fixed in -20°C methanol for 5 min, washed in PBS, and incubated with normal donkey serum (NDS) or CASBLOCK (Invitrogen, San Francisco, CA) for 1 h at room temperature to block nonspecific binding. Rabbit anti-COX2 (1:200, Abcam), rabbit anti-matrix metalloproteinase 9 (MMP9) (1:1,000, Abcam), rabbit anti-laminin 332 (1:100, Abcam), rabbit anti-keratin 6 (K6) 1:1,000, Abcam), rabbit anti-keratin 10 (K10, 1:1,000, Covance, Princeton, NJ), rat anti-Ki67 (1:50, DAKO, Carpinteria, CA) antibodies or IgG controls, diluted in PBS containing 0.05% Tween-20 and 1.5% NDS, were incubated overnight at 4°C. Sections were then incubated for 1 h with either donkey anti-rabbit Dylight 549 (1:400, Jackson Immuno Inc., West Grove, PA) or donkey anti-rat Alexa Fluor 488 (1:1000, Invitrogen, Carlsbad, CA) secondary antibodies. Tissue sections were then mounted in ProLong Gold with DAPI anti-fade reagent (Invitrogen/Molecular Probes, Eugene, OR), coverslips applied and sections cured overnight. Sections were analyzed using a Leica TCS SP5 Confocal Microscope or a Zeiss 510 LSM Confocal Microscope. For immunofluorescence microscopic analysis, the parameters of expression pattern and staining intensity used to classify the reactions were scored as follows: 1 = no staining, 2 = light staining, 3 = moderate staining, 4 = intense staining. The mean of the scored data was calculated and analyzed for significance at $p < 0.05$ using the unpaired Student's t test.

RESULTS

Histopathology of mouse skin following exposure to SM

In initial studies we analyzed the effects of SM on skin histology. In control skin, the epidermis was about 3–4 layers thick, with a thin stratum corneum and a continuous sheet of

cuboidal basal keratinocytes at the DEJ. The dermis was well organized with fibroblasts scattered throughout the tissue, sitting on a robust hypodermis and an intact muscular layer (Figures 2A, 2C). SM exposure resulted in marked histological changes in the skin. Epidermal hyperplasia with keratinocytes containing pseudoinclusions or pyknotic nuclei were observed (Figures 2B, 2D). In the dermis, edema was apparent, as well as hyperplastic epidermal appendages, inflammatory cells and hemorrhagic red blood cells. The stratum corneum was thickened with evidence of parakeratosis. Four days following SM-exposure, injury beneath the wound was apparent including loss of epithelium and increased numbers of inflammatory cells in the dermis (Figures 3A, 3B). Additionally, keratinocytes beneath the wound appeared disorganized. NDH 4338 treatment stimulated keratinocyte migration and nuclei were aligned in the direction of keratinocyte migration (Figure 3C). Following SM exposure, there was a 1.7-fold increase in dermal thickness and a 3.5-fold increase in epidermal thickness (Figure 4). NDH 4338 treatment decreased dermal and epidermal thickening by approximately one third. NDH 4338 also reduced SM-induced edema and epidermal hyperplasia (Figure 4). Vehicle alone also reduced epidermal thickness by approximately 10%.

Effects of NDH 4338 on COX2 and MMP9 expression

COX2 is the rate limiting enzyme in prostaglandin biosynthesis and a marker of skin inflammation (Lee *et al.*, 2003). COX2 was not detected in control skin or control skin treated with either vehicle or NDH 4338 (Figure 5). SM was found to upregulate COX2 expression. NDH 4338 down regulated SM-induced COX2 expression (Figure 5). These data are consistent with immunohistochemical findings that COX2 was upregulated in epidermis and hair follicles of SM treated mice (Figure 6). NDH 4338 reduced SM-induced COX2 expression throughout the epidermis and dermal appendages. The immunofluorescence mean staining intensity and distribution pattern of COX2 expression in mouse skin also supported the western blot and immunofluorescent data, which showed a significant difference of COX2 expression in skin samples of control (1.3), SM exposed (3.7), and SM exposed plus NDH4338 treatment (2.3) based on 1 as no intensity and 4 as intense staining, as indicated in Table 1.

MMP9 is a marker of extracellular matrix degradation known to play a role in SM-induced injury in mouse skin (Shakarjian *et al.*, 2006). Immunofluorescence staining showed no MMP9 in control skin, but there was a strong induction of MMP9 in hyperplastic epidermis and hair follicles after SM exposure. MMP9 expression was also noted in the stratum spinosum and the basal layer (Figure 7). Following NDH 4338 treatment, a reduction in MMP9 expression was evident in the epidermis and dermal appendages.

Effects of NDH 4338 on markers of wound repair

Suprabasal differentiating keratinocytes are known to express K10 (Fuchs, 1990). We found that K10 expression in control tissue was evident in keratinocytes in suprabasal layers and in the stratum corneum, but not in basal keratinocytes or in sebaceous glands and hair follicles of hairless mouse skin (Figure 8). SM upregulated K10 expression in hyperplastic epidermis and dermal appendages. Following NDH 4338 treatment, increased K10 expression was noted throughout the suprabasal layers of the epidermis, as well as the dermal appendages.

The immunofluorescence mean staining parameters of K10 staining showed scores of 3.7, 2.8, and 3.8, in skin samples of control, SM exposed, and SM exposed plus NDH4338 treatment, respectively, based on 1 as no expression and 4 as intense staining (Table 1).

K6, a mediator of epithelial wound repair and cell migration, was constitutively expressed at low levels in control skin (Figure 9). Four days post SM exposure, an increase in K6 expression was evident throughout the hyperplastic suprabasal epidermis. NDH 4338 decreased K6 expression (Figure 9). Evaluation of the immunofluorescence mean staining intensity indicated a significant difference of K6 expression in skin samples for control (2.0), SM exposed (3.9), and SM exposed plus NDH4338 treatment (2.6), as shown in Table 1.

Ki67 is a nuclear protein expressed in proliferating cells in response to injury (Freedberg *et al.*, 2001). In control skin, only basal keratinocytes were found to express Ki67 (Figure 10). Following SM exposure, increased numbers of suprabasal keratinocytes in the hyperplastic epithelium and the dermal appendages expressed Ki67. In addition, Ki67 positive cells, which are likely inflammatory cells, were evident in the dermis (Figure 10). NDH 4338 treatment reduced the number of suprabasal and follicular cells expressing Ki67.

Laminin 332 is an essential anchoring glycoprotein found in the basement membrane of epithelial tissues; it is important in attaching basal keratinocytes to the dermis. In control skin laminin 332 was expressed as a continuous sheet beneath basal keratinocytes and lining the sebaceous glands and hair follicles (Figure 11). Treatment of mice with SM resulted in increased laminin 332 expression which appeared in thicker multilayered sheets. Laminin 332 expression was also prominent around dermal appendages including sebaceous glands and hair follicles. NDH 4338 mitigated this response in the basement membrane. The immunofluorescence mean intensity parameter analysis showed no significant difference between controls (2.0) and NDH4338 treated samples (2.3). SM injured skin had mean immunofluorescence intensity value of 3.2 (Table 1).

DISCUSSION

NDH 4338 is a bifunctional NSAID-AChEI anti-inflammatory compound designed to target SM-induced cutaneous injury by inhibiting AChE and releasing an NSAID *in vivo* (Young *et al.*, 2012). The NSAID moiety, indomethacin, is a non-selective COX inhibitor, which has been shown to display limited efficacy against SM in the mouse ear vesicant model (MEVM) (Casillas *et al.*, 2000). In porcine skin, indomethacin reduces COX-2 mediated PGE2 production, which may contribute to its protective activity against SM-induced inflammation (Zhang *et al.*, 1995). The inclusion of an anti-cholinergic drug in NDH 4338 was based on recent studies showing the potential usefulness of targeting non-neuronal cholinergic anti-inflammatory pathways for the treatment of skin diseases and vesicant-induced blisters (Grando *et al.*, 1993; Kurzen *et al.*, 2007; Tracey, 2007; Curtis and Radek, 2012). The neurotransmitter, acetylcholine and cholinergic enzymes are produced by keratinocytes and infiltrating leukocytes during inflammatory responses; the skin cholinergic system is thought to regulate keratinocyte proliferation, differentiation, adhesion, and migration (Grando, 1997; Kurzen *et al.*, 2007). NDH 4338 was first reported to show

efficacy against half mustard-induced edema in the mouse ear vesicant model (Young *et al.*, 2010; Young *et al.*, 2012). The present studies demonstrate that NDH 4338 can also protect, at least in part, the dorsal skin of hairless mice from SM-induced injury; these findings suggest that NDH 4338 may be useful as a countermeasure against vesicant-induced skin injury.

By generating PGE₂, COX2 functions as a pro-inflammatory protein in SM-induced cutaneous injury (Yourick *et al.*, 1995; Babin *et al.*, 2000; Casillas *et al.*, 2000; Wormser *et al.*, 2004; Joseph *et al.*, 2011). Previous studies have shown that SM induces the release of arachidonic acid and metabolites including prostaglandins in human keratinocytes and a mouse neuroblastoma-rat glioma hybrid cell line (Ray *et al.*, 1995; Lefkowitz and Smith, 2002). Using a skin construct model, Black *et al.* (2010) also showed increased COX2 and prostaglandin production after exposure to the half mustard, CEES. In mouse skin, SM has also been shown to induce COX2 in keratinocytes, hair follicles, dermal fibroblasts, and inflammatory cells near wound edges (Wormser *et al.*, 2004; Joseph *et al.*, 2011). The present studies show that 4 days post SM exposure, COX2 is upregulated in mouse dorsal skin. COX2 is known to play a critical role in skin inflammation. Signaling via PGE₂ and its receptor induces vascular hyperpermeability and dermal edema (Rundhaug and Fischer, 2008; Zaroni *et al.*, 2012; Morimoto *et al.*, 2014). Our findings that inhibition of COX2 by NDH 4338 was associated with reduced edema and inflammation are consistent with a role for prostaglandins in vesicant-induced skin injury.

SM-induced toxicity is associated with reduced adhesion of basal keratinocytes and detachment of these cells from the basement membrane zone (Ruhl *et al.*, 1994; Graham *et al.*, 2005; Saladi *et al.*, 2006). Epidermal keratins are essential for maintaining skin integrity including basal keratinocyte adhesion; mutations in epidermal keratins result in perturbation of filament assembly, which can lead to skin blistering diseases (Fuchs, 1995; Uitto *et al.*, 2007). *In vitro* studies have shown that mustard vesicants induce aggregation of keratins 5 and 14; this can alter keratin filament assembly and contribute to keratinocyte detachment from the basement membrane (Dillman *et al.*, 2003; Hess and FitzGerald, 2007). Previous studies reported a significant reduction in expression of K10 in SM treated mouse skin 3 days post exposure, and increased K10 expression in the hyperplastic epidermis 14 days post exposure (Joseph *et al.*, 2011). Similarly, we found reduced expression of K10 in the suprabasal layer of hyperplastic epidermis 4 days after SM exposure; this was blocked by NDH 4338 indicating wound repair. The wound marker, K6, was also strongly expressed in SM exposed skin at this time. NDH 4338 caused a reduction of K6 in treated mouse skin providing additional support for enhanced wound repair. Moreover, the strong signal for Ki67 in SM exposed skin indicates keratinocyte hyperproliferation in both suprabasal and basal layers in response to injury; Ki67 positive cells were restricted to basal keratinocytes in SM injured skin after NDH 4338 treatment. This observation is similar to the reduction of Ki67 expression observed after closure of excisional wounds (Usui *et al.*, 2005). These observations indicate that NDH 4338 promotes wound healing and keratinocyte differentiation.

One of the most visible characteristics of SM-induced human cutaneous lesions is severe blister formation resulting from the separation of the epidermis from the dermis (Smith *et*

et al., 1995). Earlier work on the cutaneous genetic blistering disease, epidermolysis bullosa, showed that this is due to mutations in the adhesive basement membrane glycoprotein, laminin 332 (Nakano *et al.*, 2002). SM has also been shown to cause microvesication in mouse skin, with focal epidermal staining of laminin, resulting from the separation of the DEJ. This suggests a precise cleavage plane of the DEJ within the lamina lucida (Monteiro-Riviere and Inman, 1995). Laminin 332 has been reported to be upregulated and expressed at the migrating leading edge of SM wounded mouse skin (Chang *et al.*, 2009). Since laminin 332 is an essential anchoring filament attaching basal keratinocytes to the basement membrane in the lamina lucida, we analyzed its expression as a marker for basement membrane zone wound repair. We found thick multilayered expression of laminin 332 in the basement membrane zone, and more focal staining around edematous dermal appendages in SM-injured skin. These data are consistent with studies showing a multilayered and partly disrupted laminin 332 sheet in aged, sun-exposed injury, and in UV-irradiated skin models (Paeon *et al.*, 2007; Ogura *et al.*, 2008). MMPs are key effectors of extracellular matrix remodeling and are thought to contribute to the impairment of the basement membrane and degradation of its components including laminin 332, and type IV and VII collagens during injury (Ogura *et al.*, 2008). In this regard, inhibition of MMPs and other proteinases enhances basement membrane formation in UVB irradiated human skin and human skin construct models (Fisher *et al.*, 1996; Ogura *et al.*, 2008; Amano, 2009). Our studies showed that laminin 332 was more organized in NDH 4338 treated skin indicating reduced damage to the basement membrane zone. We also observed reduced expression of MMP9 in NDH 4338 treated SM injured skin. Consistent with previous findings (Vogt *et al.*, 1984; Shakerjian *et al.*, 2010), these data suggest that MMP9 plays a role in SM-induced basement membrane restoration after NDH 4338 treatment.

Inflammation is a complex process involving many cell types, cytokines, chemokines, proteases, and other factors that mediate wound repair. Our data show that NDH 4338 improves wound repair following SM-induced skin injury. Wound repair was evident by the reduction of skin edema, enhanced skin re-epithelialization, a fully differentiated epidermis, and a well-structured basement membrane zone following NDH 4338 treatment of SM wounded skin. This was associated with reduced expression of COX2 and MMP9. Additional studies will help to further characterize the anti-inflammatory mechanism of action of NDH 4338 and its effects on wound repair in the SM induced skin injury model.

Acknowledgments

This research was supported by the CounterACT Program, National Institutes of Health Office of the Director (NIH OD), and the National Institute of Arthritis and Musculoskeletal and Skin Diseases (NIAMS), grant number U54AR055073. Its contents are solely the responsibility of the authors and do not necessarily represent the official views of the federal government. This work was also supported in part by National Institutes of Health grants P30ES005022, and R01EY09056. Appreciation is extended to Linda Everett of EOHSI for editorial assistance.

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Abbreviation List

AChEI	acetylcholinesterase inhibitor
CEES	2-chloroethyl ethyl sulfide
COX2	cyclooxygenase 2
DEJ	dermal-epidermal junction
K6	keratin 6
K10	keratin 10
LM332	laminin 332
MMP9	Matrix Metalloproteinase 9
NSAID	Non-steroidal anti-inflammation drug

SM sulfur mustard

Highlights

- Bifunctional anti-inflammatory prodrug (NDH4338) tested on SM exposed mouse skin.
- The prodrug NDH4338 was designed to target COX2 and acetylcholinesterase.
- The application of NDH4338 improved cutaneous wound repair after SM induced injury.
- NDH4338 treatment demonstrated a reduction in COX2 expression on SM injured skin.
- Changes of skin repair markers are associated with NDH4438 treatment on SM injury.

Figure 1.
Chemical structure of the bifunctional anticholinergic NSAID compound NDH 4338. The drug combines two anti-inflammatory moieties (indomethacin and a choline bioisostere, 3,3-dimethyl-1-butanol), attached via an aromatic ester-carbonate linkage.

Figure 2.

Structural changes in hairless mouse skin following exposure to SM. Histologic sections, prepared 4 days after exposure to air (Ctl) (panels A and C) or SM exposure (panels B and D), were stained with H&E. Panels A and B, scale bar = 200 μ m; panels C and D, scale bar = 20 μ m. Panels C and D are enlargements of the black dotted squares shown in panels A and B, respectively. dermis (D), epidermis (E), hair follicle (HF), pyknotic nuclei (P), sebaceous glands (S), stratum corneum (SC).

Figure 3.

Effects of NDH 4338 on SM-induced skin injury. Histological sections prepared 4 days after exposure to SM, SM plus vehicle, or SM plus NDH 4338, were stained with H&E. The dotted line denotes the dermal/epidermal junction. The arrow designates the leading edge of the migrating keratinocytes. Scale bar = 20 μ m.

Figure 4.

Effects of SM exposure on skin thickness. Skin section, prepared 4 days after exposure to air (Ctl) or SM, were analyzed for dermal (panel A) and epidermal (panel B) thickness. Ctl (open bars), SM (black bars), SM plus vehicle (Veh)(gray bars), SM plus NDH 4338 (striped bars). Data are presented as the mean $\pm$ SE ($n = 20$), * $p < 0.001$, compared to control tissues; + $p < 0.001$, compared to SM exposed tissues.

Figure 5.

Effects of NDH 4338 on SM-induced alterations in COX2 expression in mouse skin. Pooled samples from each group were analyzed by Western blotting using a COX2 antibody. Lane 1, untreated control; lane 2, vehicle alone; lane 3, NDH 4338 alone; lane 4, SM; lane 5, SM plus vehicle; lane 6, SM plus NDH 4338. GAPDH expression was used to ensure equal loading of proteins on the gels.

Figure 6.

Effects of NDH 4338 on SM-induced COX2 expression in mouse skin. COX2 was visualized by confocal microscopy using a secondary antibody conjugated to Dylight 549 (red). Control mouse skin (panel A), SM (panel B), SM plus vehicle control (panel C), and SM plus NDH 4338 (panel D). Nuclei were counterstained with DAPI (shown in blue), scale bar = 20 μm.

Figure 7.

Effects of NDH 4338 on SM-induced MMP9 expression in mouse skin. MMP9 was visualized by confocal microscopy using a secondary antibody conjugated to Dylight 549 (red). Control mouse skin (panel A), SM (panel B), SM plus vehicle control (panel C), and SM plus NDH 4338 (panel D). Nuclei were counterstained with DAPI (shown in blue), scale bar = 20 μ m.

Figure 8.

Effects of NDH 4338 on SM-induced alterations in K10 expression in mouse skin. K10 was visualized by confocal microscopy using a secondary antibody conjugated to Dylight 549 (red). Control mouse skin (panel A), SM (panel B), SM plus vehicle control (panel C), and SM plus NDH 4338 (panel D). Nuclei were counterstained with DAPI (shown in blue), scale bar = 20 μm .

Figure 9.

Effects of NDH 4338 on SM-induced alterations in K6 expression in mouse skin. K6 was visualized by confocal microscopy using a secondary antibody conjugated to Dylight 549 (red). Control mouse skin (panel A), SM (panel B), SM plus vehicle control (panel C), and SM plus NDH 4338 (panel D). Nuclei were counterstained with DAPI (shown in blue), scale bar = 20 µm.

Figure 10.

Effects of NDH 4338 on SM-induced alterations in Ki67 expression in mouse skin. Ki67 was visualized by confocal microscopy using a secondary antibody conjugated to Alexa Fluor 488 (shown in red). Control mouse skin (panel A), SM (panel B), SM plus vehicle control (panel C), and SM plus NDH 4338 (panel D). Nuclei were counterstained with DAPI (shown in blue), scale bar = 20 μ m.

Figure 11.

Effects of NDH 4338 on SM-induced expression of laminin 332 in mouse skin. Laminin 332 was visualized by confocal microscopy using a secondary antibody conjugated to Dylight 549 (red). Control mouse skin (panel A), SM (panel B), SM plus vehicle control (panel C), and SM plus NDH 4338 (panel D). Nuclei were counterstained with DAPI (shown in blue), scale bar = 20 μ m.

Table 1

Immunofluorescence mean staining parameters in mouse skin.

Treatment	COX2	K10	K6	Laminin332
Control (n=8)	1.3 ± 0.3 ⁺	3.7 ± 0.3 ⁺	2.0 ± 0.0 ⁺	2.0 ± 0.0 ⁺
SM (n=10)	3.7 ± 0.2 [*]	2.8 ± 0.2 [*]	3.9 ± 0.1 [*]	3.2 ± 0.2 [*]
SM+NDH4338 (n=10)	2.3 ± 0.2 ⁺	3.8 ± 0.2 ⁺	2.6 ± 0.2 ^{*+}	2.3 ± 0.1 ⁺

Staining intensity: 1 = no staining, 2 = light staining, 3 = moderate staining, 4 = intense staining. Data are presented as the mean ± SE (n = 8–10),

^{*} p 0.05, compared to control tissues;

⁺ p 0.05, compared to SM exposed tissues.