PROTEASE INHIBITORS PREVENT MICROVESICATION IN SULFUR MUSTARD WOUNDS IN HUMAN SKIN EXPLANTS

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Summary

Sulfur Mustard is a vesicant (blistering) agent that was widely used in WWI, and was more recently used in Iraq by Saddam Hussein. Because the agent is relatively easy to manufacture and weaponize, US Intelligence estimates consider it as a significant terrorist threat. The mechanisms by which sulfur mustard creates injury on skin is thought to involve proteases (and likely other inflammatory agents), which illustrates significant similarity in the biochemistry of the damage mechanisms with some types of chemical and thermal burns.

Exposure of skin to sulfur mustard (also often called HD or SM) is thought to disrupt the balance between basement membrane protein synthesis by keratinocytes and their degradation by proteinases. This disturbance causes a loss of adherence between epidermis and dermis due to more net protein degradation than synthesis. A possible way to restore balance is to diminish or stop protein degradation. Proteinases that are thought to be involved in the degradation of the basement membrane proteins and extracellular matrix proteins (ECM) are serine proteinases (elastase) and matrix metalloproteinases (MMPS). Measurements have been made that substantiate the elevation of inflammatory cytokines, IL-1α, IL-1β, GM-CSF, TNFα and IL-6, as well as elevated MMP-9 shortly after SM exposure (Sabourin et al., 2002).

The objective of this study was the assessment of the effects of two inhibitors of MMPS, ilomastat and doxycycline, and an inhibitor of serine proteases, alpha-1 protease inhibitor (α1-PI), on damage to organ cultured human skin explants following exposure to HD vapor. As the MMP-inhibitor BB94 has previously shown to prevent epidermal-dermal separation in HD-exposed human skin pieces, this compound is included in the study as a reference (Mol et al., 2000). Ilomastat, doxycycline and BB94 are broad-spectrum MMP inhibitors; ilomastat and BB94 are hydroxamate-based compounds, and doxycycline is a semi-synthetic structural isomer of the tetracycline family. α1-PI is a natural serine protease inhibitor; one of its targets is neutrophil elastase, which might be involved in the activation of MMPS and direct degradation of membrane proteins and ECM molecules.

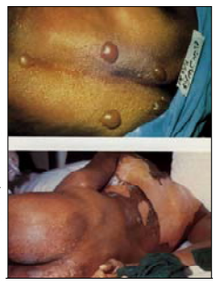


Figure 1. (above) Vesicant injury after sulfur mustard exposure. Blisters on the back 16 h after SM exposure and desquamation (buttocks) and epidermolysis (back) 5 days after SM exposure (from Willems, Annals Med Militar Belg, 1989).

Materials and Methods

The test materials, ilomastat, doxycycline and α1-PI, were supplied by Quick-Med Technologies. BB94 was kindly provided by British Biotech, Oxford, UK. Keratinocyte basal medium (KBM; Clonetics) was obtained from Biowhittaker, Verviers Belgium. HD was synthesized by the Bioorganic Chemistry Branch of TNO/Prins Maurits Laboratory and has a purity of > 97%. Human mammary skin was obtained from cosmetic surgery with informed consent of the patient.

Human mammary skin was exposed to saturated HD vapor at 25° C for five minutes using a vapor cup device (Mol el al., 1991). HD vapor exposed skin pieces of 0.25 cm^2 were floated with the dermal side down in KBM supplemented with $CaCl_2$ at a final concentration of 1.4 mM (1 ml medium/well of a 12 well cluster plate). The medium was supplemented with the indicated concentrations of the test compounds. The skin pieces were incubated at 37° C in an atmosphere of 6% $C0_2$ in air for 48 h (Varani et al., 1995).

Ilomastat was dissolved at 8 mg/ml in DMSO and then diluted 100 times in KBM to a final concentration of 80 µg/ml (1% final concentration of DMSO). α1-PI was dissolved in KBM at 2.5 mg/ml. Doxycycline was dissolved in MilliQ water at 4.6 mg/ml and then diluted 100 times in KBM to a final concentration of 46 µg/ml. BB94 was dissolved in DMSO and diluted 100 times in KBM to the final concentration of 2 µg/ml.

For histological evaluation, human skin pieces were fixed overnight in 2% para-formaldehyde in PBS and then changed to 70% ethanol until embedding in paraffin. Three semi-serial sections of each specimen were stained with hematoxylin/eosin and examined qualitatively by light microscope for blister formation and cellular necrosis in the epidermis. Photographs were taken of a representative section of three skin samples from each group.

Table 1 (at right) shows doses of sulfur mustard and associated effects, specific to topical (skin) exposure from vapour phase (sulfur mustard gas). Information from: Medical Defense Against Mustard Gas: Toxic Mechanisms and Pharmacological Implications, Bruno Papirmeister, Alan Feister et. Al., CRC Press, 1991.

Sulfur Mustard doses and severity

Dose		latency period	effect	recovery period
50 mg*min/m^3	Lower limit for injury	4-12 h	mild erythema; lower	negligible
100-300 mg*min/m^3	Mild	4-8 h	erythema, itching and sensitivity to touch	1-5 days
1000-2000 mg*min/m^3	IC_{50}	3-6 h	severe erythema followed at 12+ h by severe blistering	incapacitating injury: weeks to months
10,000 mg*min/m^3	LC_{50}	1-3 h	rapid severe erythema, followed at 3+ h by severe blistering and systemic intoxication	injury for survivors, lengthy recovery.

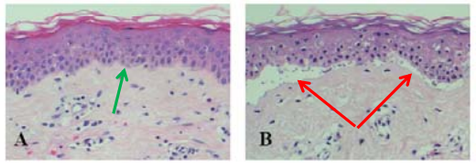


Figure 2, A and B. (right) Effects of Sulfur Mustard on human skin explants. A is unexposed skin showing healthy epidermis and dermis, with tight junction at basement membrane (BM, indicated by green arrow). B is a sulfur mustard exposed skin explant sample, showing picnotic cells in epidermis, as well as microvesication (gap indicated by red arrows) at BM.

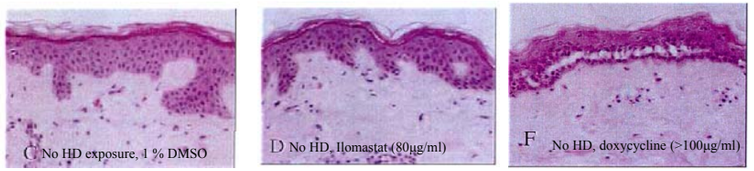
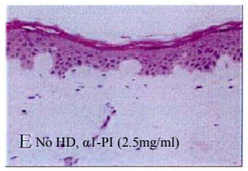


Figure 3, images C through F. (above and right) Effects of tested agents on unexposed skin explants. Doxycycline did not cause damage at a concentration of 46 µg/ml, but did induce damage to BM at concentrations >100 µg/ml. The other agents at use concentrations did not cause any skin damage.



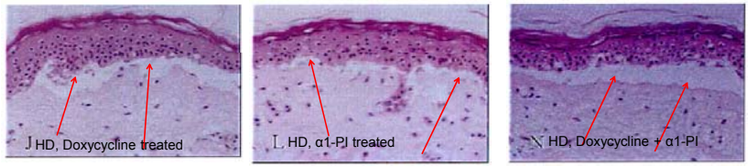


Figure 4, images J, L, N. (below) Effects of treatment with the agents on HD-exposed skin explants. Doxycycline (J) did not prevent microvesication at 46 µg/ml. A1-PI also did not provide any protection but did induce damage to BM at concentrations >100 µg/ml. The other agents at use concentrations did not cause any skin damage.

Results

Effect of HD on human skin. As shown in **figure 2**, untreated control human skin cultured for 48h has a normal appearance (**A**). Exposure of human skin to saturated vapor of HD at 25° C for 5 min results in clear epidermal damage with pyknotic nuclei and microvesication after a culture period of 48h (**B**).

Effects of the test materials on untreated control skin. As shown in **figure 3,** the presence in the culture medium of 1% MilliQ water, 1% DMSO, ilomastat (80 µg/ml), or doxycycline(46 µg/ml) had no effect on the morphology of normal skin after a culture time of 48 h. It was observed that doxycycline at a higher concentration (~100 µg/ml in KBM) caused a rupture of the epidermis above the basal cells. Skin that had been in culture medium supplemented with α1-PI (2.5 mg/ml) for 48 h showed an appearance in the epidermis slightly different from normal. A widening of the intercellular space between the epidermal cells was observed. The simultaneous presence in the culture medium of α1–PI with doxycycline augmented this phenomenon, whereas at α1–PI in combination with ilomastat did not alter the histological appearance of the skin.

Effects of the test materials on HD-exposed skin. The presence in the culture medium of 1% MilliQ water (**Figure 4),** doxycycline (46 µg/ml; **Figure 4J**), or α1-PI (2.5 mg/ml; not shown) had no beneficial effect on epidermal-dermal separation or on epidermal necrosis in HD-exposed skin. Combined treatment of skin with α1-PI and doxycycline caused no positive result on the observed damage after HD-exposure (**Figure 4L**). In contrast, epidermal-dermal separation is completely prevented when ilomastat (80 µg/ml) was added to the culture medium (Figure C). This effect is also observed when ilomastat is combined with α1-PI (2.5 mg/ml). The results obtained with ilomastat are identical to those obtained when another hydroxamate-based MMP-inhibitor, BB94, was added to the culture medium at a concentration of 2 µg/ml.

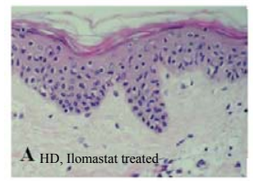
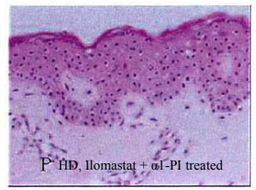


Figure 5, Ilomastat treatments. Images A (left) and P (right). Ilomastat alone as well as in combination with α1-PI was effective at suppressing microvesication in the human skin explant. Use concentrations were 80µg/ml ilomastat and 2.5mg/ml α1-PI, administered immediately after HD exposure of the tissue.



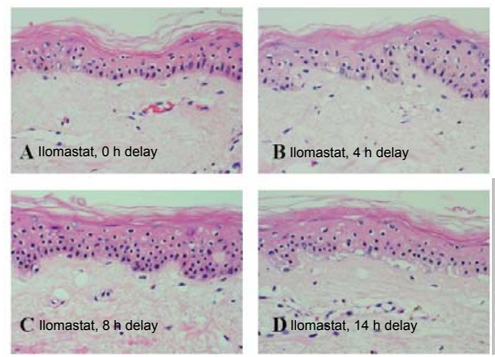


Figure 6: A through D. Ilomastat was applied at delay times of 0h (A),I 4h (B), 8h (C), and 14h (D). All times were effective at suppressing micro-vesication except the 14h delay in administration of ilomastat (all doses at 80µg/ml concentration). Ilomastat was not effective in preventing epidermal cell death at any of the tested time points.

Conclusions

Loss of the attachment of epidermal cells to the basement membrane is postulated to be a specific cause of sulfur mustard (HD)-induced vesication of the skin. Excessive proteolytic activity has been suggested to play a major role in this vesication due to an altered balance between production and degradation of ECM proteins and cell membrane proteins. Since matrix metalloproteinases (MMPs) and serine proteases (elastase) are probably involved in degradation of basement membrane proteins, two inhibitors of MMPs and one inhibitor of serine proteases were tested on their effectiveness in preventing epidermal-dermal separation. The compounds were tested in an *ex vivo* human skin model, consisting of skin pieces that were exposed *ex vivo* to saturated vapor of HD and subsequently organ cultured for 48 h. Histological evaluation of the cultured skin explants showed microvesication and extensive epidermal necrosis in skin that was not treated with a protease inhibitor. The presence in the culture medium of the hydroxamate based MMP inhibitor, ilomastat, at a concentration of 80 µg/ml completely prevented HD-induced microvesication in the human skin pieces, whereas the other MMP inhibitor, the tetracycline derivative doxycycline, was not found to be effective at the concentration of 46 µg/ml. Also, the serine protease inhibitor, α1–PI, (2.5 mg/ml) appeared to be ineffectual in preventing microvesication in the utilized *ex vivo* model of human skin. None of the tested compounds alleviated the epidermal necrosis. It is concluded that the MMP inhibitor ilomastat might be a good candidate compound to suppress the development of HD-induced blisters on human skin.

1. Medical Defense Against Mustard Gas: Toxic Mechanisms and Pharmacological Implications, Bruno Papirmeister, Alan Feister et. Al., CRC Press, 1991.
2. Sabourin CL, Danne MM, Buxton KL, Casillas RP, Schlager JJ. Cytokine, chemokine, and matrix metalloproteinase response after sulfur mustard injury to weanling pig skin. *J Biochem Mol Toxicol*. 2002;16:263-272.

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