Aus dem Walther-Straub-Institut
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Vorstand Prof. Dr. med. Thomas Gudermann

In vitro Untersuchung von epigenetischen Modifikationen nach Schwefel-Lost Exposition in Endothelvorläuferzellen

In vitro investigation of epigenetic modifications after sulfur mustard exposure in early endothelial cells

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Thilo Simons

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Berichterstatter:	Priv.-Doz. Dr. med. Kai Kehe
Mitberichterstatter:	Prof. Dr. Jürgen Behr Prof. Dr. Oliver Peschel
Mitbetreuung durch den promovierten Mitarbeiter:	Priv.-Doz. Dr. med. Dirk Steinritz
Dekan:	Prof. Dr. med. dent. Reinhard Hickel
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(Thilo Simons)

Abstract

Alkylating agents such as sulfur mustard (S-Lost, mustard gas, SM, HD, bis(2-chloroethyl)sulfide, Yperite), predominantly used by military forces, and related chemotherapeutic drugs, e.g. chlorambucil (Cbl, trade name: Leukeran), are known to cause severe cellular damage without being fully understood on a molecular level. The discovery of sulfur mustard and its use as a chemical weapon was followed by the detection of associated, medically useful toxicological properties, which paved the way for the development of alkylating chemotherapeutics. Used since the beginning of the 20^{th} century, SM has been deployed in several conflicts around the world. It can lead to severe tissue damage - in particular dermal, mucosal, ocular and respiratory lesions - followed by impaired wound healing. While investigations of the acute toxicity have been the main focus up to now, explanations of long-term effects remain widely obscure. Increased understanding of the molecular mechanisms involved might help to identify new potential targets that could improve the general therapy of victims and the development of specific therapeutics. The ability of SM to cause lasting harm (e.g. cancer) and degenerative tissue damage after even a single exposure might - among other pathological processes - be explained by the involvement of underlying epigenetic modulations. The scientific field of epigenetics comprises alterations and modifications in gene expression that are not structurally changing the DNA sequence itself, but affecting chromatin organization as well as maintenance by e.g. DNA methylation and modifying histone patterns. SM is mainly used as a liquid or aerosolic chemical weapon agent (CWA) when dispersed, and directly interacts with tissues it comes into contact with first. As well as the aforementioned tissues, this interaction also involves corresponding small blood vessels. Blood vessel malformation (e.g. cherry hemangioma) is a frequent observation after SM exposure. Blood vessel formation depends on regeneration and immigration of endothelial cells. Early endothelial cells (EEC), which are known to play an important role in the formation of granulation tissue and the process of wound healing, provide a rational *in vitro* model to analyze the molecular toxicology of SM. After determining the lethal concentrations of sulfur mustard and assessing specific doses (0.5 μM, 1.0 μM) at which EECs are affected but maintain their cell division and proliferation abilities, we analyzed selected epigenetic modulators; a potential up- and downregulation of epigenetically relevant genes was examined. The EECs were tested for histone dimethylation (H3-K9, H3-K27, H3-K36), histone acetylation (H3-K9, H3-K27, H4-K8) and global DNA methylation (5-mc, 5-hmc). The changes were investigated over 24 hours and for up to 4 cell passages. Moreover, we were able to assay abdominal-thoracic skin samples from a laboratory worker who accidentally exposed himself to a high dose of SM, which we received one year after the initial exposure subsequent to a corrective surgical procedure. The results of our *in vitro* study clearly show changing epigenetic patterns over time, which partly coincide with the findings from the human skin samples exposed short-term to SM. Histone modifications generally remained fewer and DNA methylation increased significantly. Future investigations should focus on the confirmation of these results under *in vivo* conditions and include possible therapeutic interventions, e.g. DNA methyltransferase inhibitors, to prevent or reverse these effects.

Zusammenfassung

Alkylierende Substanzen, wie das militärisch genutzte S-Lost (Senfgas, SM, HD, bis(2-chloroethyl)sulfide, Yperite) und verwandte Chemotherapeutika, wie z.B. Chlorambucil (Cbl, Handelsname: Leukeran), sind bekannt dafür, schwere zelluläre Schäden zu verursachen, wobei die zu Grunde liegenden molekularen Grundlagen noch zu großen Teilen unverstanden sind. Der Entdeckung von S-Lost und dessen Einsatz als chemischer Kampfstoff folgte die Feststellung verschiedener, medizinisch nützlicher Eigenschaften, was den Weg für die Entwicklung alkylierender Chemotherapeutika ebnete. SM wurde seit Beginn des 20. Jahrhunderts weltweit in verschiedenen Konflikten verwendet. Sein Einsatz kann zu schweren Gewebeverletzungen mit nachfolgend gestörter Wundheilung führen, die besonders dermale, mukosale, okuläre und respiratorische Läsionen nach sich ziehen. Während bisher besonders Untersuchungen in Hinblick auf die akute Toxizität im Fokus standen, mangelt es weiterhin an Erklärungen für die auftretenden Langzeiteffekte. Ein genaueres Verständnis der beteiligten Mechanismen könnte neue mögliche Angriffspunkte in Hinblick auf eine Verbesserung der derzeitigen, allgemeinen Therapiemöglichkeiten aufzeigen und die Entwicklung spezifischer Therapeutika ermöglichen. Die Fähigkeit von SM schwerwiegende Folgeerkrankungen (z.B. Malignome) und degenerative Gewebeschäden nach bereits einer einzigen Exposition zu verursachen, könnte unter anderem durch in Zusammenhang stehende epigenetische Prozesse zu erklären sein. Das Wissenschaftsfeld der Epigenetik umfasst Veränderungen und Modifikationen in der Genexpression, die nicht die Basenabfolge der DNA selbst, sondern die Organisation und molekulare Integrität des Chromatins betreffen, z.B. in Form von DNA-Methylierung sowie der Modifizierung von Histonmustern. SM ist ein hauptsächlich in flüssiger beziehungsweise aerosolischer Form vorliegender, chemischer Kampfstoff und interagiert unmittelbar mit Geweben mit denen es primär in Kontakt kommt. Dies beinhaltet, neben den zuvor erwähnten, auch korrespondierende kleine Blutgefäße. Blutgefäßfehlbildungen (z.B. tardive Hämangiome) werden häufig nach SM-Exposition beobachtet. Blutgefäßneubildung hängt von Regeneration und Immigration von endothelialen Zellen ab. Frühe endotheliale Zellen, die bekannterweise eine wichtige Rolle in der Neubildung von Granulationsgewebe und dem Prozess der Wundheilung besitzen, liefern ein rationales *in vitro* Modell für die Analyse der molekularen Toxizität von SM. Wir bestimmten zunächst die letalen Konzentrationen für S-Lost und die genauen Dosisbereiche (0,5 μM, 1,0 μM) in denen die EEC´s zwar Schaden davontrugen, aber die Fähigkeit zur Zellteilung und Proliferation erhalten blieb, wonach epigenetische Modulatoren analysiert wurden. Wir untersuchten mögliche Up- und Downregulationen epigenetisch-relevanter Gene. Die EEC´s wurden auf Histondimethylierung (H3-K9, H3-K27, H3-K36), Histonacetylierung (H3-K9, H3-K27, H4-K8) und globale DNA-Methylierung (5-mc, 5-hmc) getestet. Im Folgenden wurden Veränderungen über 24 Stunden und bis zu 4 Zellpassagen analysiert. Außerdem waren wir in der Lage eine abdominell-thorakale Hautprobe von einem im Rahmen eines Arbeitsunfalls mit einer hohen SM-Dosis exponierten Patienten mit in unsere Untersuchungen einzubeziehen, die ungefähr ein Jahr nach Primärkontamination durch eine korrektive, chirurgische Maßnahme gewonnen wurde. Die Ergebnisse unserer *in vitro* Studie zeigen deutlich sich verändernde epigenetische Muster im Zeitverlauf je nach S-Lost Konzentration, z.B. traten Histonmodifikationen seltener auf, wohingegen DNA-Methylierungen insgesamt signifikant zunahmen. Dabei stimmen sie in weiten Teilen mit den Ergebnissen der intoxikierten Hautprobe überein. Zukünftige wissenschaftliche Untersuchungen sollten auf die Bestätigung der Ergebnisse unter *in vivo*-Konditionen abzielen und mögliche therapeutische Ansätze, wie z.B. DNA-Methyltransferase-Inhibitoren, einbeziehen, um den beobachteten Effekten vorzubeugen oder sie zu behandeln.

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Original Research Articles Presented in this Thesis

Steinritz, D., Schmidt, A., Balszuweit, F., Thiermann, H., **Simons, T.**, Striepling, E., Bölck, B., Bloch, W. (2016). Epigenetic modulations in early endothelial cells and DNA hypermethylation in human skin after sulfur mustard exposure. *Toxicology Letters*, Vol. 244, pp. 95-102.

Simons, T., Steinritz, D., Bölck, B., Schmidt, A., Popp, T., Thiermann, H., Gudermann, T., Bloch, W., Kehe, K. (2018). Sulfur mustard-induced epigenetic modifications over time - a pilot study. *Toxicology Letters*, Vol. 293, pp. 45-50.

Further Publications

Steinritz, D., Schmidt, A., **Simons, T.**, Ibrahim, M., Morquet, C., Balszuweit, F., Thiermann, H., Kehe, K., Bloch, W., Bölck, B. (2014). Chlorambucil (nitrogen mustard) induced impairment of early vascular endothelial cell migration - Effects of α-linolenic acid and N-acetylcysteine. *Chemico-Biological Interactions*, 219, 143-150.

Bloch, W., **Simons, T.**, Steinritz, D., Schmidt, A., Bölck, B. (2014). Stability and time course of epigenetic changes by S-/N-alkylating substances. *Wehrmedizinische Monatszeitschrift* Vol. 58, pp. 316-317.

Bloch, W., **Simons, T.**, Steinritz, D., Schmidt, A., Bölck, B. (2017). Sulfur mustard and epigenetics. *Wehrmedizinische Monatszeitschrift*, Vol. 61, p. 9

1 Introduction

1.1 History and Actuality

1.1.1 Sulfur Mustard - History

Alkylating agents have been subject of intense research since their discovery in the 19th century and frequent usage from the beginning of the 20th century onward [7, 19]. Yet there are many unsolved questions in relation to their severe toxicological effects on different cell types [107, 114, 118]. The vesicant SM has by now a long history as an excruciating military weapon. It incapacitates enemies rather than killing them, and by the later development of nitrogen mustard-based derivates like chlorambucil (Cbl, trade name: Leukeran) it found entrance into modern medicine [19, 73, 93]. Cbl gained further importance in scientific research when used for example as a mutagen for molecular mapping and structure-function correlations of genomic regions [117]. SM was first synthesized by Despretz, supposedly in 1822, followed by further characterization by Niemann and Guthrie in 1860 [82, 138]. The German chemists LOmmel and STeinkopf shaped the well-known acronym LOST [82, 102]. Haber pushed the large-scale production of SM, which led to its initial military usage - or more precisely to its first heavy application - during the First World War (WWI) by German forces in Belgium in 1917, resulting in about 400,000 casualties [82, 138]. Despite the international restriction of chemical warfare by the Geneva Protocol in 1925, it was continuously produced and brought into action in various conflicts around the world, among these the Rif War 1920-1927, Egypt against North Yemen 1963-1967 and the Iran-Iraq War 1980-1988, whereupon it was specified in the List of Schedule 1 substances within the Chemical Weapons Convention (CWC) in 1993 as a substance with no other use than in warfare [18, 21, 43, 99]. In 1997 the CWC agreement entered into force, mandating destruction of all chemical weapons by 2007 [93]. But nearly 100 years after its first use, mustard gas - in some reports referred to as “king of the battle gases” - is still a current threat in many parts of the world [45, 69].

1.1.2 Sulfur Mustard - Actuality

During the Syria crisis, which started in 2012, large amounts of chemical warfare agents, including SM, were confirmed as being part of the armory of the Syrian military [128]. In private briefings to weapons experts in 2013, White House officials said, analysts concluded, that Syria possessed about 300 metric tons of sulfur mustard and unfortunately these weapons had probably been brought into action since the conflict had started [45, 145]. In 2014 the allegedly last stockpiles of the chemical armory were hydrolyzed on board the Cape Ray - a vessel equipped with mobile chemical decontamination systems - in the Mediterranean Sea, and then shipped to Finland, Britain, Germany and the US for final combustion of the hydrolysates according to media reports of The Washington Post and The New York Times [46, 63, 13, 108]. Despite these attempts it is likely that SM was released in Avdiko, Syria, causing at least 3 deaths and several casualties displaying the typical burning signs of this agent [129]. In 2015 the BBC reported about an investigation from the Organization for the Prohibition of Chemical Weapons (OPCW) which revealed

that SM was most likely utilized in Marea, Syria during an attack in August of the same year [14]. The news agency Reuters and the Security Council of the United Nations (UN) reported about the evidence of the deployment of SM in Aleppo, Syria in September 2016 based on laboratory analysis of blood samples obtained from victims [95, 120]. As well as active deployment, another frequently forgotten problem is still-undetected stockpiles that continue to pose a threat, as shown by the non-occupational exposure of 43 people in Qiqihar, in Northeast China's Heilongjiang province, in 2003. When several barrels of SM leaked at a construction site that was used by Japanese military forces and then abandoned in 1945, several different kinds of typical injuries were reported [136]. Similar cases occurred in the area of Bornholm, a Danish island in the Baltic Sea, where approximately 200,000 tons of SM had been discarded and were sporadically and accidentally retrieved by local fishermen, who then were exposed because of shell leakage [150]. Similar processes of ocean dumping were registered in the Atlantic Ocean as depicted in Figure 1.1.

Figure 1.1: Ocean dumping of containers with sulfur mustard [68].

Although representing an oily liquid, SM was mistakenly called mustard "gas" in the past due to its aerosolic condition when dispersed by explosion [75]. SM's degradation and reactivity depend heavily on its environment. In water, the process of hydrolysis and dissolution of SM depends on the salt content, temperature, pH value, pressure, density and viscosity [67]. The half-life in seawater at e.g. 5°C was stated to be 175min [36]. But it was proven that SM can resist degradation over longer periods as well which enhances the risk of an eventual exposure [67]. Besides, SM and its degradation products were found to be extremely stable in dry matrices for up to four

years, so that an eventual exposure needs to be feared [98, 89]. Residues of SM were found at autopsy even seven days after initial exposure in several tissues, particularly in fat, skin with subcutaneous fatty tissue and in the brain [32]. The varying half-life of SM *in vivo* has been observed in several studies [78, 12]. To avoid the aggregation of SM, early decontamination of the affected environment and as part of the medical approach remains highly important. A detailed operating plan regarding verification of exposure, treatment options and approach to decontamination was for example published by Steinritz et al. [135]. The limited half-life *in vivo* of pure SM and its converted degradation products on the one hand, and delayed chronic health effects on the other, pose questions about the molecular mechanisms, enacting long-term modifications, that SM is capable of inducing [135].

1.1.3 Chlorambucil - Medical Use of Sulfur Mustard Derivates

Even though SM itself currently has no medicinal use, it has been serving as a prototypic alkylating drug for research into the development of various medical treatments. During the Second World War (WWII), a ship that carried mustard gas shells exploded in Bari, Italy in 1943 [102]. Survivors were observed to develop leucopenia after their rescue; this led to the idea of utilizing SM, specifically its derivates, to treat diseases that induce a hyperplastic bone marrow and lymphocytosis [56]. In the 1940s Goodman and Gilman had already begun clinical trials with nitrogen mustard derivates for the treatment of lymphoma, and found them to be effective; this resulted firstly in the development of aliphatic mustards like mechlorethamine and then of less toxic aromatic nitrogen mustards like chlorambucil, which was approved in 1957 [8, 34, 52]. Other chemically related chemotherapeutics with differing toxicological properties like cyclophosphamid, ifosfamid, melphalan and bendamustin were developed as well within a few years and are still of clinical relevance [137]. Cbl´s relatively slow reactivity and the possibility of its oral administration have contributed since then to its distinct indications and wide usage [106]. As part of the World Health Organization's List of Essential Medicines it has been benefiting patients with different diseases including leukemia, Hodgkin´s and non-Hodgkin´s lymphoma, Morbus Waldenstrom, ovarian carcinoma, autoimmune and inflammatory illnesses such as psoriasis, ulcerative keratitis or rheumatoid arthritis; in addition to that, it helped to establish organ transplantation [2, 9, 98, 125, 140]. On the other hand it is also known to act like a human mutagen, potentially causing other neoplastic diseases [93]. Severe side effects include unwanted vascular tissue damage and organ dysfunction [24].

1.2 Clinical Picture of Mustard Gas Intoxication and Side Effects of Chlorambucil

1.2.1 Sulfur Mustard - Most Affected Organs

After contact with SM, vesicating lesions especially in dermal, mucosal, ocular and respiratory tissues as well as systemic effects and, in the long-term, even psychological symptoms were noticed, depending on the site, time course and concentration of the exposure [115, 133]. Sulfur mustard-related injuries can take months to heal; cells with high proliferative and metabolic capacity are particularly vulnerable [48, 98]. Most commonly affected are skin and eyes as well as the respiratory tract [69].
Normally starting a few hours after an exposure - with shorter latency at very high doses and relative to the cell type - incapacitating effects begin to manifest with an eventual death rate of less than 4%, but requiring elaborate medical intensive care

Table 1.1: Sulfur mustard-induced clinical symptoms - most common effects on often affected organs. Synopsis from [3, 11, 70, 35, 44, 48, 65, 75, 101, 104, 110, 111, 112, 147, 151].

dermal symptoms

acute
vesication/blistering, tautness, erythema/reddening, itching, edema

delayed
ulceration, epidermal necrosis, subepidermal location, disseminated bullae

long-term
wound healing disorders, pigmentation disorders, cancer development

ocular symptoms

acute
blepharospasm, burning, foreign body sensation, photophobia, lacrimation, blindness

delayed
ulceration, scarring, corneal thinning, vasculitis of the cornea and conjunctiva

long-term
aberrant vessel formation, neovascularization, chronic blepharitis, lipid and cholesterol/amyloid deposition, limbal stem cell deficiency, perilimbal conjunctival ischemia, blindness, mustard gas keratopathy

pulmonary symptoms

acute
cough, bronchial obstruction, dyspnoe

delayed
hyperreactive airways, bronchiectasis, pseudomembrane formation, emphysema

long-term
interstitial fibrosis, bronchiolitis obliterans, decreased total lung capacity, tracheobronchomalacia, air trapping, recurring chronic pulmonary infections, mustard lung

and specific burn management in order to prevent dehydration, electrolyte imbalance and infection [18]. An important problem in terms of treatment is that patients might barely complain about any symptoms in the first moments after contact so that exposure can remain undetected leading to delays in diagnosis and therapeutic measures. SM is systemically incorporated at a very low dose and was shown to compromise multiple organ systems when less than 10% of the dermal body surface is affected [75]. For skin cells the onset of symptoms can take from a few up to 24 hours [135]. The feature of being very soluble in fat rather than in water makes human skin and associated cell types like hair follicles and sweat glands particularly vulnerable. While about 80% of SM evaporates after skin contact, the remaining 20% is absorbed within approximately two minutes [70]. Symptoms may emerge as sunburn-like erythema with tautness and reddening, which can escalate to ulceration, epidermal necrosis together with subepidermal location, disseminated bullae and hypo- or hyperpigmentation, notably in the intertriginous areas [112]. Development of skin cancer as a long-term consequence was reported [110]. Ocular lesions are also common with a varying frequency of up to 75-90% [11]. The unprotected eyes represent the most sensitive organ showing a general latency of symptoms of about 1-4 hours [3, 135]. Keratitis and anterior uveitis as part of ocular symptoms can include the sensation of dry eyes with foreign body sensation, photophobia and lacrimation [11, 48, 101]. Clinical findings comprise corneal thinning with irregularities, scarring, ulceration, vasculitis of the cornea and conjunctiva [11, 48, 101]. Partially these effects heal within 1-2 weeks, but in the long-term aberrant vessel formation, neovascularization and chronic blepharitis, together with lipid and cholesterol/amyloid deposition, are dreaded complications [3, 11]. Conjunctival ischemia and vasculitis caused by chronic inflammation and pathological metabolic processes regarding corneal innervation support the notion of SM's chronic manifestation [11, 65]. In some patients, eye damage undergoes a clinically silent phase before initiating a second cascade, leading in the worst cases to organ malformation and blindness [65]. A comparative study estimated the rate of pulmonary involvement at about 42.5%, where the most common acute symptoms appeared to be cough and dyspnea [111]. Acute pulmonary injury can lead via bronchial obstruction to respiratory failure with life-threatening consequences like asphyxia [147]. Lung-specific cell types like type I and type II alveolar epithelial cells show partial loss of cell membranes and disorganized microvilli [151]. Macroscopically, tracheobronchomalacia and air trapping often appear in high-resolution computerized tomography [104]. In the medium to long-term, victims can suffer from hyperreactive airways, bronchiectasis, pseudomembranes, emphysema and recurring chronic pulmonary infections resulting in a decreased total lung capacity [48]. Fibrotic processes often cause chronic diseases such as bronchiolitis obliterans and interstitial fibrosis [147]. A follow-up examination of 197 cases 10 years after a single heavy exposure revealed chronic bronchitis in almost 60% of the patients to be the leading late sequela [35]. Due to the diverse and complex respiratory disorders which often consist of different individual characteristics the term "mustard lung" was established [44]. Beyond these feared organ-specific effects, the risk of infection is generally increased by a dose-dependent internal suppression of immunocompetent cells and barrier disorders of the primarily affected tissue [53]. Especially at high-dose levels systemic cytotoxic effects - involving fast dividing cells like those of the intestinal mucosa and cells of the hematological system - and genotoxic and mutagenic impacts were observed [18]. Long-term consequences are recurrent infections and malignancies like acute myeloid leukemia and acute lymphocytic leukemia, but also weakness, insomnia, headache, loss of appetite, nausea, vomiting and defective spermatogenesis, that emerge partially even years later [53, 119, 136]. Progeny of SM-affected soldiers

showed a significantly elevated overall manifestation of physical abnormalities and disorders [1].

1.2.2 Sulfur Mustard - Case Report of Accidental Exposure

First-hand case reports of SM documenting the acute and chronological course of symptoms are rare, since intoxication mainly occurs in wars and terroristic attacks with very limited opportunities for follow-up examination or cohort stratification. During our research we were able to examine a case of accidental exposure by analyzing skin samples from an intoxicated male laboratory worker, who was involved in a process of chemical plastic production. Until the incident it was not known that SM was formed as an intermediate product in that specific process making a certain type of plastic. Despite his wearing of several layers of clothing including his laboratory coat, and his immediate change of apparel after noticing a leak in a tube, SM was able to penetrate and affect his skin. Dermal contact with the agent was not perceived at that moment and first symptoms in the central abdominal region began approximately 30 minutes after the incident, resulting in an approximately 2 cm measuring erythema. The fact his left forearm was affected remained temporarily unnoticed.

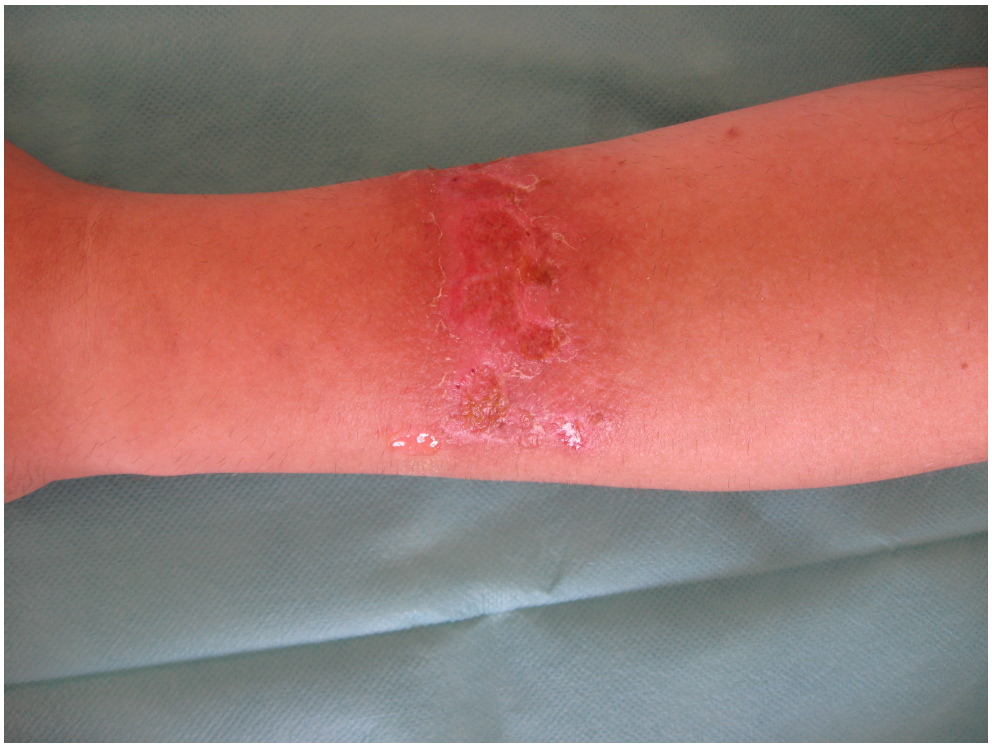

Figure 1.2: Right forearm 11 days after accidental exposure to sulfur mustard [123].

Both regions showed severe skin detachment over time. Even decontamination with Previn® solution, treatment with hydrogen peroxide, cortisone ointment and repetitive disinfectant measures could not prevent grave blistering and ulceration with the necessity of extensive surgical debridement and split-skin grafting two weeks after

the event, covering parts of the abdominal and ventral thoracic region.

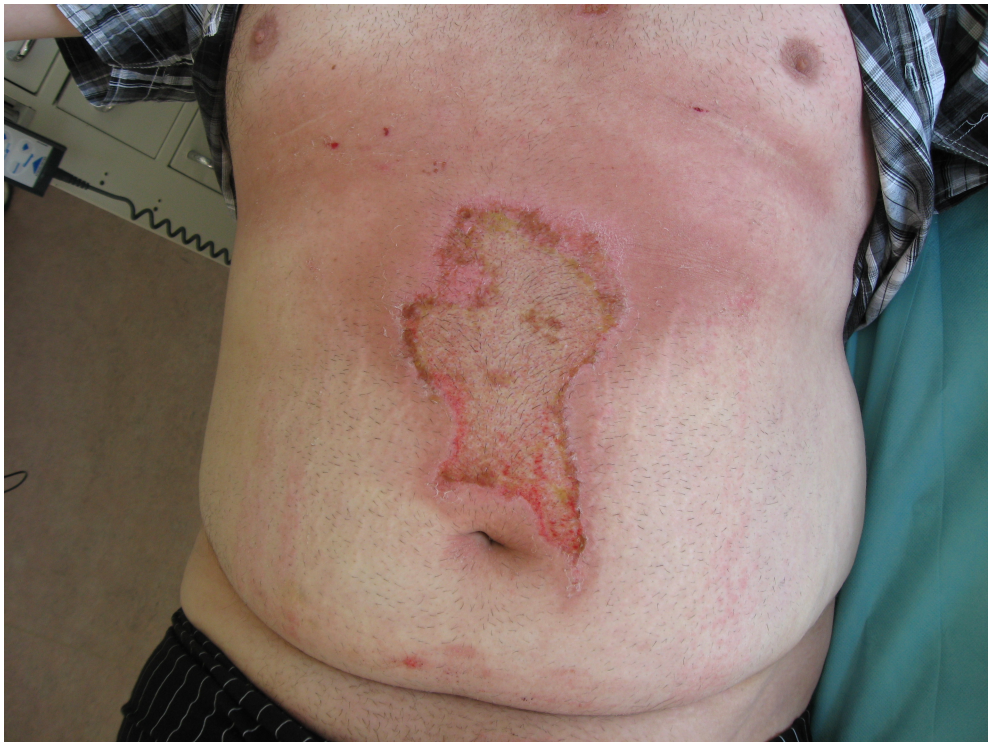

Figure 1.3: Abdominal region 10 days after accidental exposure to sulfur mustard [123].

The full report was published by Schmidt *et al.* [123].

1.2.3 Chlorambucil - Side Effects

Clinical presentation of patients during therapy with medically applied alkylating agents is different, but several systemic effects appear similar [126]. While a dose-dependent reversible myelosuppression in the context of treatment of leukemia is intended, the effects on other organ systems complicate continuous usage and limit the therapy [103]. Cbl treated patients generally show milder side effects since the chemical compounds are less toxic; furthermore, production and application is thoroughly supervised. Like many other chemotherapeutic drugs it potentially causes diverse unwanted side effects in the gastrointestinal and central nervous system; it can also lead to unwanted effects on skin and hair, damage to the liver and the reproductive system as well as lung fibrosis and anemia [5, 144]. Boosted activation of peripheral blood leucocytes can cause vascular tissue damage and organ dysfunction [24]. Chronic consequences include severe diseases. In several studies during 1981-1987 it was shown that the incidence of leukemia among with Cbl treated polycythemia vera patients was significantly higher and rose with increased pharmaceutical dose and duration of therapy [15]. A similarity between Cbl and SM is their ability to affect endothelial cells [66, 122]. Their importance is further discussed in section 1.4.

1.3 Appearance, Chemical Structure and Molecular Effects

Alkylating agents can be characterized by their general property of being „capable of covalently attaching an alkyl group to a biomolecule under physiological conditions“[8]. SM is a colourless or pale yellow and at room temperature oily liquid with a high lipid solubility and low volatility; it is usually deployed via aerosol, and can in its impure form have a scent of mustard or garlic and a darkened colour [18, 75, 113]. SM has a melting point of 14°C and a vapor pressure of 0.11mmHg at 25°C. It is heavier than air and accumulates under moderate conditions in air layers near the ground [82]. SM is a classified group 1 carcinogen [98]. Its toxic potential is attributable to its high reactivity [4]. The molecule is pictured in figure 1.4.

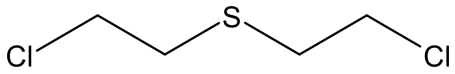

Figure 1.4: Structural formula of sulfur mustard created with ChemSketch.

Major consequences on a molecular level are loss of cell integrity and disruption of transcription and replication [70, 102]. Enhanced inflammatory response and initiation of necrotic and apoptotic cell death can be triggered [53, 71, 109]. These effects complicate wound healing and tissue repair.
Cbl is a chemotherapeutic drug applied orally in tablet form. It is less electrophilic than SM, containing a nitrogen atom instead of the sulfide, and is - due to the aromatic ring's electron withdrawing capacity - less reactive, which permits a longer half-life in serum and facilitates oral administration [25, 50]. The molecule is pictured in figure 1.5.

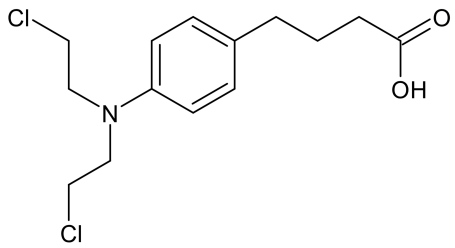

Figure 1.5: Structural formula of chlorambucil created with ChemSketch.

Cbl is about 98% plasma-bound and becomes rapidly metabolized to phenyl acetic acid mustard (PAAM) [121]. Its cytostatic and immunosuppressive properties are based upon abnormal base pairing and cross linkage of DNA and RNA strands with subsequent interference with replication especially during the S-cyclus leading to apoptosis [47, 146]. Therefore, fast-dividing cells including endothelial precursors are at particular risk [122].

1.4 Epigenetics and Endothelial Cells

1.4.1 Epigenetics - General Information

Epigenetics is a relatively new scientific field that has been treated with increasing interest in recent years. It was established to describe processes that connect the previously separately-treated areas of developmental biology and genetics [57]. Alongside the well known term ‘genotype‘, the epigenetic equivalent ‘epigenotype‘ was coined [57]. While the genotype remains in a fairly stable configuration, the epigenotype, as a second entity responsible for gene expression, is more dynamic and inconsistent, reacting to cellular stimuli or metabolic requirements [42, 74]. These stimuli include, for example: physical exercise, diet and exposure to chemicals [86, 90]. However, despite being modifiable, environmental influences may sometimes lead to enduring epigenetic changes and change of gene expression through epigenetic modifications seems to be inheritable as well [55, 105, 143]. The transmission of epigenetic patterns to descendants is called transgenerational inheritance [86]. In terms of diagnosis and treatment, difficult illnesses like cancer or autoimmune diseases, but also neurodegenerative and psychological disorders, behavioral plasticity and addiction have been related to epigenetic aetiologies [20, 90]. The term ‘epigenetics‘ comprises alterations in gene expression that are not related to the base sequence of DNA itself, but rather to chromatin organization and maintenance [60]. This includes histone posttranslational modifications, DNA methylation and the involvement of RNA-based mechanisms [79, 83]. Figure 1.6. shows a graphical illustration of these three mechanisms of gene regulation.

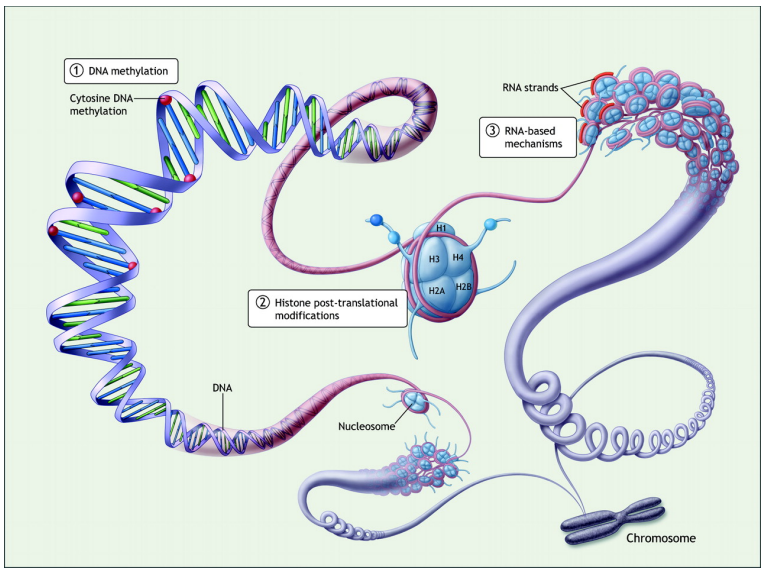

Figure 1.6: Epigenetic mechanisms of gene regulation.
(1) DNA methylation (depicted as red balls) involves the covalent modification of cytosine in the context of CpG dinucleotides to define the ‘fifth base of DNA‘ 5-methyl-cytosine. (2) Posttranslational modifications of the histone amino terminal tails (depicted as light and dark blue balls) are myriad and can importantly affect the physical properties and higher-order compaction of chromatin. (3) RNA-based mechanisms have recently emerged as important regulators of chromatin structure and gene expression (depicted as red strands coating chromatin) [83].

Histones, as the principal structural proteins of eukaryotic chromosomes, help to package the DNA to fit inside the nucleus [23, 130]. Histone modifications are key components of transcriptional regulation, modulating the chromatin structure [31, 96]. More precisely, correlations of modifications regarding chromatin structure and function play a significant role in directing the level of chromatin compaction and mediating functional pathways influencing the readout of distinct regions of the genome [42]. Histone modifications are also conducive to genome stability by signalling DNA damage and initiating the assembly of repair foci [88]. Furthermore, they enable a histone code with the potential to extend the information of the genetic code [64]. Common histone modifications include acetylation and methylation, which were found to contribute to control of stem cell maintenance, differentiation and function [62, 96]. Relevant enzymes hereof are histone methyltransferases (HMTs), histone acetyltransferases (HATs) and deacetylases (HDACs) [49, 77]. The balance between methylation and acetylation status plays an important role in gene accessibility [97]. While histone acetylation leads to a relaxed chromatin state and thereby enables transcriptional activation, deacetylation is linked to chromatin compaction and transcriptional inactivation [94]. Overall, histone methylation is the major component of chromatin modification; it is controlled and maintained by SET domain proteins [76]. Histone methylation is generally associated with transcriptional repression and occurs mainly on histones H3 and H4 [27, 54]. H3 and H4 are part of four core histone proteins forming an octamer as part of the nucleosome [61]. Changes in H3 and H4 under the influence of SM were specifically investigated in our research. Another important epigenetic modification is DNA methylation. Mainly occurring at the C-5 position of cytosine within CpG dinucleotides, methylated cytosine is considered to be the principal epigenetic tag that in regulatory regions probably prompts the inactivation of the corresponding gene like an on-off switch [87, 94]. The result is the creation of 5-methylcytosine (5-mc). This process is described by the term ‘hypermethylation‘ [84]. DNA methyltranferases (DNMTs) are key enzymes which promote this modification. Further oxidation by TET-proteins leads to 5-hydroxymethylcytosine (5-hmc), which is mainly found in neuronal and embryonal stem cell chromatin [22]. 5-hmc was also found to be associated with labile nucleosomes [94]. The oxidation of 5-methylcytosine to 5-hydroxymethylcytosine is considered to be an initial step in DNA demethylation pathways [149]. These complexes are often referred to as 5^{th} (5-mc) and 6^{th} (5-hmc) DNA bases [116]. DNA methylation fulfills different functions in mammalian organisms. Heavily methylated genes are less likely to be translated into mRNA [86]. If normal, DNA methylation contributes to chromosomal stability, X-chromosome inactivation, imprinting and compartmentalization of chromatin [37]. It is involved in the developmental and transcriptional regulation of tissue-specific gene expression and contributes to gene silencing [33, 73, 148]. Phenotypic variations and disease susceptibility are associated with it [148]. DNA methylation patterns can guide specific proteins to corresponding target sites [139]. Serving also as epigenetic memory, DNA methylation status is regarded to be more stable than other epigenetic modifications [90]. Aberrant DNA methylation can have severe consequences, such as disruption of imprinting and x-chromosome inactivation, as well as erroneous cloning [37]. It is an important factor in the development of various diseases especially cancer [139]. Tumor cells frequently show global genomic hypomethylation leading to the increased activity of oncogenes, while promotor regions of tumor suppressor genes exhibit a state of hypermethylation [86]. It’s been proposed that similar changes occur in cardiovascular and immune disease [37]. In addition to the aforementioned modifications, RNA-based mechanisms are involved in epigenetic control. This includes interfering on the level of chromatin

and on the post-transcriptional level as they form a powerful surveillance system dealing with suspect transcription events [58, 84]. It has been proposed that there exist various layers of RNA-based regulation including non-coding RNA (ncRNA) [51]. NcRNA, structurally subdivided into small (sncRNA) and long (lncRNA) entities, can as active regulators modulate gene transcription and thereby influence gene expression [51, 141]. These units contribute to mechanisms such as gene silencing, DNA demethylation, RNA interference, imprinting and gene co-suppression [86]. Recent scientific discoveries underlined their importance as key players in the process of carcinogenesis [38]. The increasing evidence of the contribution of ncRNA to the genesis and progression of many other diseases and their feature to be potentially approached with drug therapy makes them therapeutically interesting [141]. However, the exact roles of ncRNA are not yet completely understood, which still complicates the development of therapeutics [141].

1.4.2 Epigenetics and Endothelial Cells

Several studies indicate the involvement of epigenetic pathways in the control of vascular endothelial function. It is presumed that epigenetic mechanisms act as mediators of environmental influences on vascular endothelial gene expression through the regulation of factors which can predispose to vascular diseases [79, 83]. Epigenetic mechanisms were found to substantially contribute to the regulation of endothelial progenitor cell (EPC) tasks [40]. EPCs are considered crucial for successful neovascularization and wound healing [29]. They are for example involved in the process of neovascularization in different diseases like retinopathy, vascular diseases and myocardial ischemia [72]. While endogenous endothelial progenitor cells are important to initiate the body´s own vasculogenesis, therapeutically applied exogenous endothelial progenitor cells can be of benefit to patients, as e.g. initiators of revascularization after ischemic injury [40]. Human endothelial colony-forming cells (ECFCs), pretreated *ex vivo* with epigenetic drugs, triggered enhanced formation of capillary-like networks and accelerated restoration of perfusion, while maintaining responsiveness to signals from the environment, which also may prevent blood-vessel overgrowth [39]. Current research also includes various therapeutic approaches in diabetic wound healing and peripheral arterial disease (PAD) [41].

1.4.3 Endothelial Cells and Sulfur Mustard

Endothelial cell migration-mediated vasculogenesis and angiogenesis are vital factors in the healing process of ischaemic tissues and complex wounds caused by toxic chemical substances [85, 132]. While many investigations about mechanisms and measures in the process of wound healing after contact with SM until now focused on keratinocytes and fibroblasts, newer studies confirmed alkylating agents impair the integrity of blood vessels and their regeneration [122, 124]. SM leads to altered endothelial morphology and increased capillary leakage [6]. Impairment of tissue repair might be aided by the dysfunctional and reduced migration and proliferation of endothelial cells. Considering the importance of endothelial progenitor cells for wound healing, EECs present a reasonable *in vitro* approach [142]. The effects of SM on endothelial cells are subject to continuous study. In 1996 Dabrowska *et al.* showed that SM severely damages endothelial cells and underlined the important differences in the apoptotic and necrotic processes involved, as well as their correlation with intracellular ATP-levels [26]. Morphologic differences of control endothelial cells and cells exposed to 500 μM are depicted in figure 1.7.

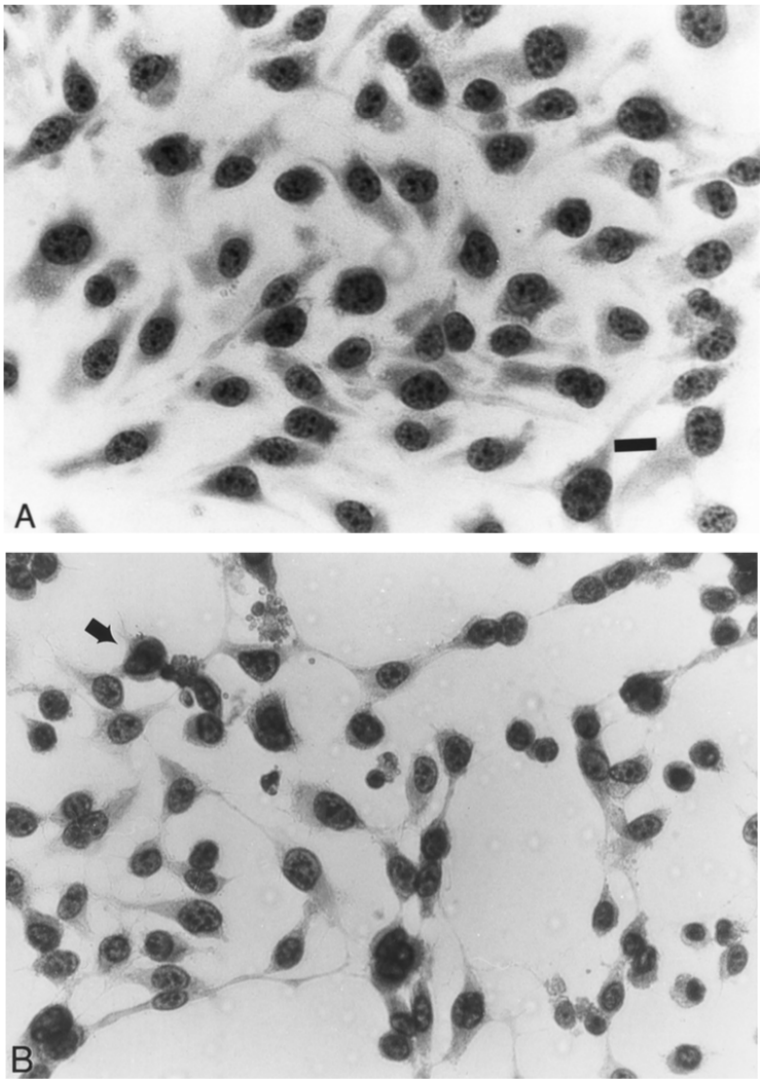

Figure 1.7: Micrographs of Wright–Giemsa-stained endothelial cells. (A) Control endothelial cells. (B) Cells exposed to 500 μM SM, after 5 hrs. With an increase of chromatin condensation (arrow) and a partly irregular cell array. [26].

Recent research suggests that SM has negative effects on vascularization and endothelial tube formation e.g. in the pathophysiology of SM-based eye damage [100, 131].

1.4.4 Sulfur Mustard and Epigenetics

Recent studies underlined the importance of epigenetic mechanisms in the development of SM-induced cellular dysfunction and long-term effects [74, 105]. Epigenetic modifications were supposed to be responsible for chronic consequences like the clinical picture of the mustard lung [59]. However exact patterns of modified DNA-related molecules could barely be identified; a comprehensive understanding of the epigenetic role of short and long-term effects remained absent [81, 102].

2 Objectives of the Presented Thesis

The primary purpose of this study was to confirm the epigenetic contribution of SM-caused pathology with a special focus on its long-term effects and to identify potential therapeutic targets that enable the development of causal preventative and symptomatic therapies.

The main questions the thesis addresses are:

- To which specific doses of SM is it possible to expose EECs so that they maintain the ability of several cell divisions?
- Which epigenetic modulators and patterns are modified in affected cells and what are the differences to non-exposed cells?
- Do different patterns persist over time, and if so, do they stay stable for several cell divisions?
- Are there any congruent results between *in vitro* and *in vivo* affected cells?
- What are potential epigenetic targets for therapeutical intervention?

3 Publications

3.1 Epigenetic Modulations in Early Endothelial Cells and DNA Hypermethylation in Human Skin After Sulfur Mustard Exposure

Steinritz, Dirk; Schmidt, Annette; Balszuweit, Frank; Thiermann, Horst; Simons, Thilo; Striepling, Enno; Bölck, Birgit; Bloch, Wilhelm, Toxicology Letters, 26 of February 2016, Vol. 244, pp. 95-102

In order to approach the investigation of epigenetic changes that potentially occur in affected early endothelial cells, it was necessary to figure out at which concentrations to treat these cells with SM while maintaining their capacity for subsequent *in vitro* cultivation. The first step was to investigate the cell viability under influence of SM through XTT-based colometric assay. The LC_{50} was reached at 11,7 μM. Remarkably, to achieve an appropriate survival rate among early endothelial cells for up to 4 passages - as further described in section 3.2 - we also had to establish very low concentrations of 0.5 μM (~1/20 of LC_{50}) and 1.0 μM (~1/10 of LC_{50}). Higher concentrations of 23.5 μM (~2 x LC_{50}) and 50 μM (~5 x LC_{50}) were set in comparison in the short-term groups 24 hours post exposure. In this study 78 genes related to epigenetic pathways in EECs were analyzed *in vitro*. Besides, we were able to compare the results to skin samples from an accidentally to SM exposed laboratory worker. Applied investigation techniques comprised epigenetic chromatin modification enzymes PCR array, DNA extraction, detection of global DNA methylation *in vitro* and DNA methylation in human skin tissue. The results were statistically analyzed. SM significantly changed various of the examined epigenetic modulator genes and global DNA methylation status in the affected skin samples compared to non-exposed control skin. In the *in vitro* tests genes were analyzed that encode e.g. DNA methyltranferases and histone methyltransferases, acetyltransferases and deacetylases. Overall 37 genes remained unaltered whereas 41 underwent either up- or downregulation. The low doses caused more changes in gene regulation (22 and 29) than the high doses (10 and 10). DNA methylation status showed a significant increase. Remarkably, after contact with high doses of SM (23.5 μM, 50 μM) DNA methylation appeared to decrease again in comparison to the low doses (0.5 μM, 1 μM). The skin samples partly showed an even stronger increase of 5-mc than the *in vitro* tests but the range of variation of DNA methylation levels strongly increased as well, so that statistical significance was not achieved [127, 133]. The key findings can be summarized as follows: (I) Under influence of SM complex regulation patterns of epigenetic modulators could be validated and (II) global DNA methylation increased *in vitro* and *in vivo* in different cell types.

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Epigenetic modulations in early endothelial cells and DNA hypermethylation in human skin after sulfur mustard exposure

Dirk Steinritz[a,b,*], Annette Schmidt[a,c], Frank Balszuweit[a], Horst Thiermann[a], Thilo Simons[a], Enno Striepling[d], Birgit Bölck[c], Wilhelm Bloch[c]

[a] Bundeswehr Institute of Pharmacology and Toxicology, 80937 Munich, Germany
[b] Walther-Straub-Institute of Pharmacology and Toxicology, Ludwig-Maximilians Universität München, 80336 Munich, Germany
[c] Department of Molecular and Cellular Sports Medicine, German Sports University, 50933 Cologne, Germany
[d] Department of Handsurgery, Plastic and Microsurgery, Burn Center, BG Trauma Hospital Hamburg, 21033 Hamburg, Germany

HIGHLIGHTS

- Sulfur mustard induced epigenetic modulations were explored in early endothelial cells.
- 78 Genes related to epigenetic pathways and global DNA methylation were investigated.
- SM exposure resulted in complex epigenetic perturbations and DNA methylation *in vitro*.
- Increased DNA methylation was found in SM exposed human skin one year after exposure.
- Epigenetic modulations may be responsible for SM-induced long-term health effects.

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ABSTRACT

Victims that were exposed to the chemical warfare agent sulfur mustard (SM) suffer from chronic dermal and ocular lesions, severe pulmonary problems and cancer development. It has been proposed that epigenetic perturbations might be involved in that process but this has not been investigated so far.

In this study, we investigated epigenetic modulations *in vitro* using early endothelial cells (EEC) that were exposed to different SM concentrations (0.5, 1.0, 23.5 and 50 μM). A comprehensive analysis of 78 genes related to epigenetic pathways (*i.e.*, DNA-methylation and post-translational histone modifications) was performed. Moreover, we analyzed global DNA methylation *in vitro* in EEC after SM exposure as a maker for epigenetic modulations and *in vivo* using human skin samples that were obtained from a patient 1 year after an accidently exposure to pure SM.

SM exposure resulted in a complex regulation pattern of epigenetic modulators which was accompanied by a global increase of DNA methylation *in vitro*. Examination of the SM exposed human skin samples also revealed a significant increase of global DNA methylation *in vivo*, underlining the biological relevance of our findings. Thus, we demonstrated for the first time that SM affects epigenetic pathways and causes epigenetic modulations both *in vivo* and *in vitro*.

1. Introduction

Sulfur mustard (SM, bis(2-chloro-ethyl) sulfide, CAS No. 505-60-2) is a chemical warfare agent (CWA) that was first used during World War I. Later it was deployed in warfare in the Middle East resulting in a large number of victims (Ghabili et al., 2010). Exposure to this compound causes affection of skin, eyes, lungs and systemic effects (Kehe et al., 2009b). Acute dermal symptoms are most common and are characterized by erythema, burning sensation, itching, vesication, ulceration, wound healing disorder and pigmentation disorder (Graham and Schoneboom, 2013). However, most victims suffer from a plethora of chronic health effects of varying severity (Emadi et al., 2012; Ghabili et al., 2010; Kehe and Szinicz, 2005; Rowell et al., 2009). Common problems being reported in casualties include dermal symptoms, chronic

* Corresponding author at: Bundeswehr Institute of Pharmacology and Toxicology, 80937 Munich, Germany.
E-mail address: dirk.steinritz@lrz.uni-muenchen.de (D. Steinritz).

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ocular lesions, severe pulmonary problems and cancer development (Doi et al., 2011; Easton et al., 1988; Graham and Schoneboom, 2013; Hosseini-khalili et al., 2009; Rowell et al., 2009).

Although not fully understood, the pathophysiology of acute SM toxicity is much more elucidated than the chronic health effects. Several mechanisms, *e.g.*, alkylation of biological macro-molecules including DNA, excessive DNA-repair, induction of cell death, oxidative stress, activation of MMP, disturbed cell signaling -just to name a few- have been identified that contribute to acute toxicity (Bhat et al., 2000; Debiak et al., 2009; Kehe et al., 2009a; Ries et al., 2009; Rosenthal et al., 2003; Steinritz et al., 2009). However, these mechanisms cannot explain SM induced lesions that occur sometimes decades after the initial exposure (Ghabili et al., 2010; Ghanei et al., 2010), especially with respect to the fact that intact SM, its biotransformation products and protein adducts have a very limited half-life *in vivo* and are eliminated within some weeks after exposure (Barr et al., 2008; Black et al., 1997; Li et al., 2013). A meaningful pathophysiological explanation for delayed SM induced chronic health effects is lacking.

It has been proposed that SM induced chronic health effects may be associated with epigenetic perturbations (Korkmaz et al., 2008a,b) but this has not been investigated so far. "Epigenetics" refers to the temporal and spatial control of gene activity without altering the underlying DNA nucleotide sequence. Epigenetic modifications – including methylation, acetylation, phosphorylation, and ubiquitination amongst others – alter the accessibility of DNA to the transcription machinery and therefore influence gene expression. Especially aberrant DNA methylation was found to be associated with human diseases including cancer (Jones and Baylin, 2002; Robertson, 2005). Methylation of cytosine in CpG dinucleotides is probably the most important covalent modification of DNA, resulting in transcriptional repression (Bird, 2002). Post-translational modifications of histones (*e.g.*, acetylation, methylation and phosphorylation) are additional mechanism for the regulation of gene activity (Lee et al., 2010).

A plethora of proteins are known or predicted to modify genomic DNA and histones thereby regulating gene expression. Proteins mediating such modifications include DNA methyltransferases, histone acetyltransferases, histone methyltransferases, SET domain proteins, histone phosphorylating proteins, proteins for histone ubiquitination, DNA/histone demethylases and histone deacetylases. These proteins are summarized as "epigenetic modulators". There is considerable evidence suggesting that epigenetic mechanisms mediate the development of chronic health effects by modulating a plethora of genes such as expression of pro-inflammatory cytokines, interleukins, tumor suppressor genes or oncogenes (Shanmugam and Sethi, 2013). Moreover, epigenetic pathways have been linked to the control of vascular endothelial function (Fraineau et al., 2015; Lewis et al., 2014; Matouk and Marsden, 2008) and endothelial dysfunction has been observed in various cardiovascular and pulmonary diseases including Asthma, COPD and chronic bronchitis (Ives et al., 2014; Vukic Dugac et al., 2015). Remarkably, these chronic diseases are frequently observed after SM exposure (Ghabili et al., 2010; Razavi et al., 2013; Rowell et al., 2009). In this study, we exposed early endothelial cells (EEC) to different SM concentrations to explore whether SM influenced epigenetic pathways in EEC and affected global DNA methylation *in vitro*. In addition, we analyzed global DNA methylation in SM exposed human skin samples. These samples were obtained from a patient who accidentally exposed himself to a small amount of pure SM at the abdomen and thorax region and who was subjected to a surgical revision approx. one year after the accident.

2. Material and method

2.1. Cell culture and incubation with SM

Early endothelial cells (EEC) were obtained from differentiated murine embryoid bodies (for details see (Schmidt et al., 2009; Schmidt et al., 2004)) through magnetic-activated cell sorting (MACS) on day 7 after start of differentiation. MACS was performed using a mini-MACS system with MS separation columns (Miltenyi Biotec, Bergisch Gladbach, Germany) as described earlier (Dainiak et al., 2007; Schmidt et al., 2004a). The PECAM-1 positive fraction of cells was cultured on gelatine-coated dishes in DMEM, (Dulbecco's Modified Eagle medium) supplemented with 15% heat-inactivated fetal calf serum (FCS), 50 U/ml penicillin, 50 U/ml streptomycin, 200 μM L-glutamine, 100 μM β-mercaptoethanol, and 1% MEM (non-essential amino acids (GIBCO-BRL, Gaithersburg, USA)). After 2–3 weeks, these PECAM-1 positive EEC were passaged for the first time and afterwards once till twice a week. Cells were used to passage 8. Cells were handled under sterile conditions and cultivated with 5% CO_2 at 37 °C and 95% humidity. SM (purity >99%, approved by NMR) was obtained from TNO (The Hague, The Netherlands) and diluted in ethanol. For dose-finding experiments EEC were exposed to SM with concentrations ranging from 0 to 500 μM. For assessment of epigenetic changes, EES were exposed with concentrations of 0.5, 1.0, 23.5 and 50 μM SM. Controls were treated with ethanol (2.5‰) in DMEM. All SM exposure experiments were conducted at the Bundeswehr Institute of Pharmacology and Toxicology.

2.2. Cell viability

EEC were seeded in 96-well plates at a density 50,000 of cells per well and grown under standard conditions for 24 h. DMEM was removed and cells were exposed to sulfur mustard at concentrations ranging from 0 to 500 μM in DMEM. Controls were treated with ethanol (2.5‰) in DMEM. After 1 h medium was renewed and cells were grown for additional 24 h. Cell vitality was then determined using the XTT assay (Roche, Switzerland). Non-linear regression of dose-response curves was conducted using GraphPadPrism v5.01 software. 4 Independent biological experiments with 4 technical replicates were conducted.

2.3. Epigenetic chromatin modification enzymes PCR array

2.3.1. Total RNA isolation

EEC were exposed to SM (0.5, 1.0, 23.5 or 50 μM) or treated with ethanol (2.5‰) for 1 h. After 24 h total RNA was extracted from control and SM exposed EEC using the RNeasy® Mini Kit (Qiagen, Hilden, Germany), according to the manufacturer's instructions. Genomic DNA was degraded by DNase-on column treatment with RNase-free DNase set (Qiagen, Hilden, Germany). The purity of extracted RNA was assessed by measuring the optical density (OD) at wavelengths of 230 nm, 260 nm and 280 nm using a NanoDrop® ND-1000 spectrophotometer (NanoDrop Technologies, Wilmington, USA). Absorbance ratio at 260/280 nm and 260/230 nm was used to assess the purity of the RNA samples.

2.3.2. cDNA synthesis using the RT^2 first strand kit

Total RNA was transcribed into cDNA using the RT^2 First Strand Kit (SABioscience/Qiagen, Hilden, Germany) according to the manufacturer's instructions. In brief, volumes corresponding to 1 μg of total RNA were mixed with 2 μl of genomic DNA (gDNA) elimination buffer in a nuclease-free PCR tube. RNAse-free H_2O was added to a final volume of 10 μl. After thorough vortexing, the mixture was incubated for 5 min at 42 °C to degrade any gDNA in the samples. Afterwards samples were placed on ice for at least

1 min. Then, 10 µl of the reverse transcription mix was added to each tube. The mixture was gently resuspended and tubes were placed in a thermocycler (FlexCycler^2, Analytik Jena, Germany) and incubated at 42 °C for 15 min. Then, the thermocycler was immediately heated to 95 °C for 5 min to stop cDNA synthesis by inactivation of the reverse transcriptase and degradation of remaining RNA. 91 µl of DEPC–water were added to each vial containing 20 µl cDNA solution resulting in a total volume of 111 µl. Samples were stored at −20 °C until further analysis.

2.3.3. qRT-PCR using the RT^2 profiler^{TM} PCR array

102 µl cDNA solution (see Section 2.3.2), 1248 µl RNase-free water and 1350 µl 2× RT^2 qPCR SYBR® Green Mastermix (consisting of HotStart DNA Taq polymerase, PCR buffer, dNTP mix (dATP, dCTP, dGTP, dTTP) and SYBR® Green dye, SABioscience/Qiagen, Hilden, Germany) were mixed. After thorough stirring, 25 µl were transferred into each well of the RT^2 Profiler^{TM} PCR Array plate (PAMM-085Z, SABioscience/Qiagen, Hilden, Germany) containing the pre-dispensed forward and reverse primers for each individual gene. The plate was carefully sealed with an optical adhesive film and liquids in the wells were briefly spun down at 2500 rpm for 20 s (VWR PCR Plate Spinner, Darmstadt, Germany). Then, the plate was placed in a Mx3005P Cycler (Stratagene Agilent Technologies, Santa Clara, CA, USA) and qRT-PCR detection was performed using the a two-step cycling program. The first step was conducted for 10 min and 95 °C for heat activation of HotStart Taq DNA polymerase. Next, 40 cycles each with 15 s at 95 °C and 1 min at 60 °C was performed to first disassociate DNA double strands and then amplify DNA and data acquisition. Melting curves were recorded to verify specific production of a single PCR product for each individual gene and exclude the formation of primer dimers and other unspecific PCR products. Further quality controls were performed using an excel-based RT^2 RNA QC PCR Array template (http://www.sabiosciences.com/pcrarraydataanalysis.php) that allowed evaluation of CT-values of specific PCR controls measured with every PCR plate. Only data that met the control requirements (reverse transcriptase activity, positive PCR control and negative genomic DNA control) were used for further analysis. 5 housekeeping genes included in the arrays were used for normalization of data.

2.3.4. PCR data evaluation

Threshold cycle (CT) values for each well were calculated using the real-time cycler software. An Microsoft Excel®-based spreadsheet evaluation (provided by SABiosciences http://www.sabiosciences.com/pcrarraydataanalysis.php) was used to calculate the mRNA expression in SM exposure groups in relation to the ethanol control group using the $2^{-\Delta\Delta CT}$ method. Changes in gene expression were reported in a biologically meaningful way, especially for decreased gene expressions, by dividing $-1/2^{-\Delta\Delta CT}$. Expression changes > 1 had not to be converted. The cut-off for gene regulation was set to 2-fold (−2 for down-regulation and +2 for up-regulation). Microsoft Excel® was used to generate a heat map and to calculate intersections between the individual groups. Hierarchical clustering analysis was performed using Cluster 3.0 with distance measures based on the Pearson correlation and visualized using TreeView. Protein Association Network Analysis was performed using STRING (www.string-db.org/).

2.4. DNA extraction

EEC were exposed to SM (0.5, 1.0, 23.5 or 50 µM) or treated with ethanol (2.5‰) for 1 h. Cells were detached by Accutase (PAA Laboratories GmbH, Linz, Austria), followed by a centrifugation at 200 × *g* for 10 min. Cells were diluted in DMEM to 3×10^6 cells to meet the genomic DNA extraction kit (NORGEN Biotek Corporation, Canada) criteria. Genomic DNA was extracted according to the manufacturer's protocol. In brief, cells were lysed by adding 200 µl Digestion Buffer, 15 µl Proteinase K and 20 µl RNase A and subsequent incubation at 55 °C for 1 h. After cell lysis, samples were vortexed for 15 s before 200 µl Buffer SK was added and the samples were thoroughly vortexed. Then 200 µl ethanol (96%) was added. After vortexing the whole 600 µl volume per sample was transferred to a spin column assembled with a provided collection tube. Next, tubes were centrifuged at 5200 × *g* for 2 min. Afterwards, columns (with DNA bound to the resin) were washed twice with 500 µl wash solution. Finally, columns were placed in new eppendorf tubes and DNA was eluted by adding 200 µl Elution Buffer B to the resin bed and centrifugatuin at 3000 × *g* for 1 min and for an additional 2 min at 14,000 × *g*. DNA concentration was quantified by UV spectrophotometry using a NanoDrop® ND-1000 spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA) measuring the absorption at A260/280. DNA concentrations were calculated as the mean of the three runs for each sample. Samples were stored at −80 °C until further analysis.

2.5. Global DNA methylation

Global DNA methylation was assessed by determination of 5-methylcytosine (5-mC) using an ELISA-based assay (5-mC kit; Zymo Research, California, USA) following the manufacturer's instructions. In brief, a standard curve was generated based on 7 standards. Each DNA samples was adjusted with 5-mC Coating Buffer to a final DNA concentration of 1 ng/µl. 100 µl of the sample was denatured at 98 °C for 5 min using a thermocycler and was transferred on ice for 10 min. The entire sample volume (100 µl) was then used for coating a 96-well plate (1 h, 37 °C). After 3× washing with 200 µl of 5-mC ELISA Buffer, 100 µl antibody mix (consisting of anti-5-methylcytosine and the secondary antibody in 5-mC ELISA Buffer) was added to each well. Samples were incubated at 37 °C for 1 h. Plates were washed 3× with 200 µl 5-mC ELISA Buffer. 100 µl of Horseradish Peroxidase Developer were added to each well and incubated at room temperature. Absorption was measured using a plate reader (Multiskan FC, Thermo Scientific, USA) at 405 nm. 5-mC levels of all samples were normalized to ethanol controls that were set to 1.

2.6. DNA methylation in human tissue

During an accident in a chemical plant a worker exposed himself to a small amount of pure sulfur mustard at the abdomen

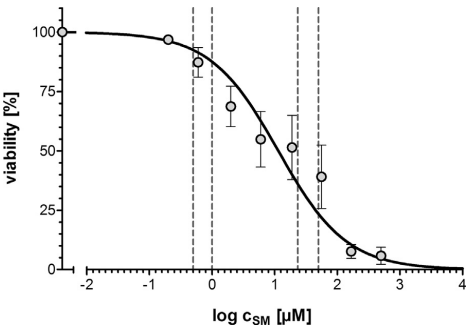

Fig. 1. Sulfur mustard cytotoxicity in EEC.
EEC were treated with SM using concentrations from 0 to 500 µM. Cell viability was assessed 24 h post exposure using the XTT assay. Gray dotted lines represent concentrations of 0.5, 1.0, 23.5 or 50 µM SM that were used in subsequent experiments. Data represent means ± SD from 3 independent experiments each with 4 technical replicates.

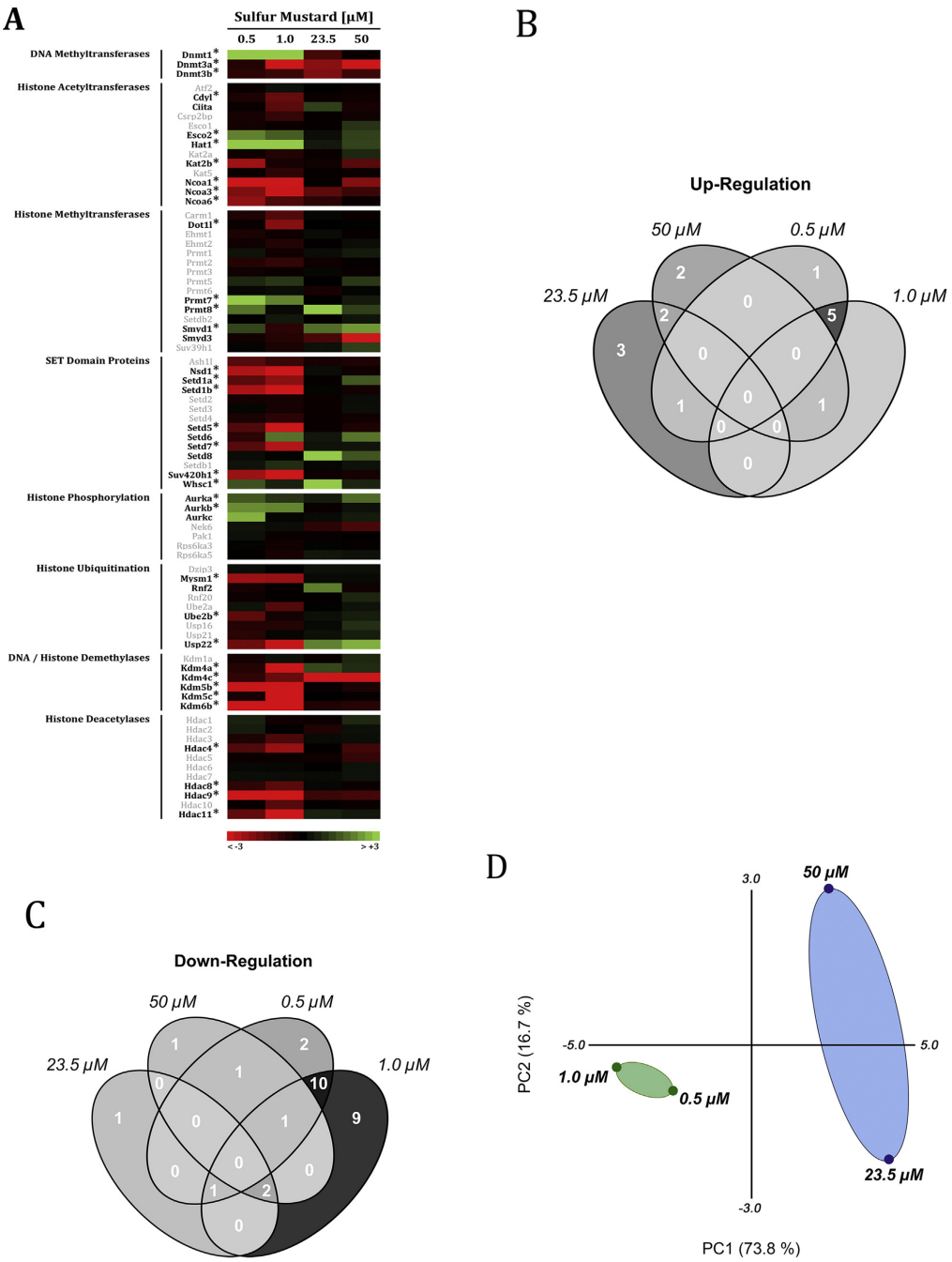

Fig. 2. Changes of epigenetic chromatin modification enzymes in EEC after SM exposure.
EEC were exposed to ethanol (2.5‰) or SM (0.5, 1.0, 23.5 or 50 µM). Changes of epigenetic modulators was assessed 24 h post exposure using a PCR array profiling the expression of key genes encoding enzymes known or predicted to modify genomic DNA and histones to regulate chromatin accessibility and therefore gene expression. The heat map (A) displays changes in gene regulation (green = upregulation; red = downregulation) compared to the ethanol control group. Proteins that exhibited a > 2 fold change in gene regulation in at least one group (41 genes) are displayed in black letters whereas unchanged genes (37 genes) are displayed in grey letters. Significant changes ($p < 0.05$) are marked with asterisks. Low dose exposure groups (0.5 and 1.0 µM SM) and high dose exposure groups (23.5 and 50 µM) exhibited distinct differences: in low

and thorax region. Due to a wound healing disorder at the abdominal site, a skin graft was performed 14 days after the incident. The thorax area healed spontaneously. Approx. one year after exposure, a surgical revision was conducted at all former exposure sites at the explicit request of the patient, who feared a tumor development. During surgery, the formerly non-operated thorax site (thorax (central)) with adjacent tissue (thorax (peripheral)) and the transplant with adjacent tissue (abdomen (peripheral)) were removed. In addition, the patient approved removal of a control skin area from the upper leg (unexposed skin) that was definitively not exposed to sulfur mustard. Skin samples from each skin area were divided into 3 samples and immediately deep frozen after removal. Tissue samples were sliced in 7 μm section thickness with a microtome (Leica CM1900, Leica Microsystems, Nussloch, Germany) and homogenized into fine powder in liquid nitrogen. 300 μl of Digestion Buffer A was added to 20 mg of the sample according to the manufacturer's protocol from the Genomic DNA Isolation Kit (NORGEN Biotek Corporation, Thorold, Canada). Slicing and Homogenation was repeated 3 times, thereby obtaining 3 technical replicates from each skin specimen. Global DNA methylation was assessed using the MethylFlash™ methylated quantification colorimetric assay (Epigentek, Farmingdalte, USA).

2.7. Statistics

Significant changes in gene expression were calculated based on a Student's *t*-test of the replicate $2^{(-\Delta Ct)}$ values for each gene in the control group and SM exposure group using a spreadsheet provided by SABiosciences (http://pcrdataanalysis.sabiosciences.com). Significance was assumed at $p < 0.05$. Means and standard deviations were calculated and significance was tested by One-Way ANOVA followed by Bonferroni's correction for multiple comparison using GraphPad Prism 5.04 (GraphPad Software). Significance was assumed at $p < 0.05$. Principle component analysis (PCA) was performed using a free-available Excel® add-in from NumericalDynamics.com (www.numericaldynamics.com).

3. Results

3.1. Sulfur mustard cytotoxicity in EEC

Exposure of EEC to different concentrations (0–500 μM) of SM and assessment of cell viability 24 h post exposure revealed a LC_{50} of 11.7 μM (Fig. 1). For further experiments SM concentrations of 0.5 (~1/20 of LC_{50}), 1.0 (~1/10 of LC_{50}), 23.5 (~2 × LC_{50}) and 50 (~5 × LC_{50}) were chosen. 3 Independent experiments, each with 4 technical replicates were conducted.

3.2. Epigenetic chromatin modification enzymes

Changes in gene regulation of epigenetic modulating enzymes in response to SM were investigated 24 h post exposure using a qPCR array. 78 Genes including DNA methyltransferases, histone acetyltransferases, histone methyltransferases, SET domain proteins, histone phosphorylating proteins, proteins for histone ubiquitination, DNA/histone demethylases, histone deacetylases were explored. From the 78 investigated genes, 37 remained unchanged in all exposure groups whereas 41 genes were up- or down-regulated > 2 fold in at least one group. Exposure of EEC to 0.5 μM SM affected the regulation of 22 genes with 7 up-regulated and 15 down-regulated genes. 29 Genes were differentially regulated at 1.0 μM SM with 23 down-regulated and 6 upregulated genes. At higher SM concentrations the number of regulated genes decreased to 10 genes at both 23.5 μM and 50 μM. 4 Genes were down- and 6 genes were up-regulated at 23.5 μM SM and 5 genes were down- and 5 genes were up-regulated at 50 μM SM. Investigated house-keeping genes (*e.g.*, actin, GAPDH) revealed no significant changes in any SM exposure group. Comparing the group mean differences by paired one-way ANOVA and Sidak correction for multiple testing revealed no significant differences between the 0.5 and 1.0 μM as well as no difference between the 23.5 and 50 μM SM exposure groups. However, a significant difference between the mean of 0.5 and 1.0 and the mean of the 23.5 and 50 μM SM exposure group was detected ($p < 0.001$). This distinctive difference was also found in a principle component analysis. Therefore, data was merged to a low dose exposure group (0.5 and 1.0 μM SM groups) and a high dose exposure group (23.5 and 50 μM groups) thereby matching similar regulated genes within the two groups. In the low dose exposure group 17 genes were differentially regulated with 12 down- and 5 up-regulated genes. In contrast, only 4 genes exhibited a differential expression in the high dose exposure group with 2 down- and 2 up-regulated genes. Obviously, more genes were affected in the low dose exposure and predominantly down-regulated. No gene showed a consistent regulation in all groups. However, Usp22 was down-regulated in low dose exposure groups whereas it was up-regulated in high dose exposure groups. SM induced down-regulation of Dnmt3a and Kdm4c was detected at SM concentration of 1.0 and above and followed a dose response relationship. A down-regulation of Ncoa3 was observed already at 0.5 μM SM while increasing SM concentrations mitigated this effect. Dnmt1, Dnmt3a, Hat1, Hdac9, Hdac11, Kdm4c, Kdm6b, Ncoa3, Prmt8, Set1b and Whsc1 revealed most pronounced changes. Results are summarized in Fig. 2 in form of a heat map (Fig. 2A), as a Venn diagram (Fig. 2B and C), as a principle component analysis (Fig. 2D) and are listed in Table S1. Fold-change values and protein clusters are given in Supplementary Table S1.

3.3. 5-Methylcytosine levels in EEC after SM exposure

5-Methylcytosine (5-mc) levels in EEC 24 h increased significantly after exposure to 1.0 and 23.5 μM SM compared to ethanol controls. After exposure to 0.5 μM SM, 5-mc increased slightly (approx. 1.2 fold), but not significantly. Both 1.0 and 23.5 μM SM resulted in an approx. 2.5 fold increase of 5-mc levels. At 50 μM an approx. 2 fold increase was observed, however, level of significance was not reached. All 5-mc levels were normalized to 5-mc levels in ethanol controls. 3 independent experiments each with 3 technical replicates were conducted (Fig. 3).

3.4. 5-Methylcytosine levels in SM exposed human tissue

Global DNA methylation (5-methylcytosine, 5-mc) was assessed in all samples. Data were normalized to control (unexposed skin) levels. Formerly exposed skin tissue from the thorax as well as potentially exposed but not primarily removed adjacent tissue revealed significant higher levels of 5-mc

dose exposure groups significant more genes were regulated (29 genes up- or downregulated compared to only 4 up- or downregulated genes within the high dose exposure group). No gene was up- or down-regulated in all groups. However, 3 genes (Dnmt3a, Ncoa3, Kdm4c) were down-regulated following a dose response relationship over three groups. Relations between each data set for up-regulated genes (B) or down-regulated genes (C) are given in the Venn diagram. Shades correspond to the number of genes per intersection. Both Venn diagrams confirm that low dose and high dose exposure groups exhibit distinct differences with regard to epigenetic pathways. A principle component analysis (D) revealed that the low dose exposure group is distinctively different from the high dose exposure group. All data represent means from 3 independent biological experiments per condition.

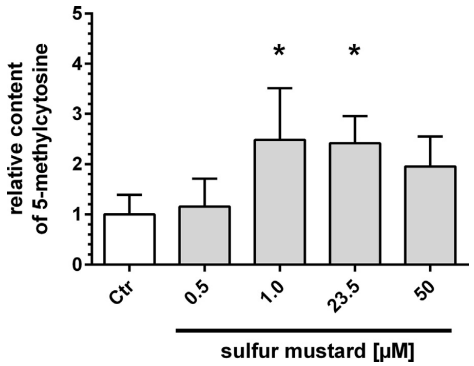

Fig. 3. Global DNA methylation in EEC.
EEC were exposed to ethanol (2.5‰) or SM (0.5, 1.0, 23.5 or 50 µM). 24 h after exposure the content of 5-methylcytosine (5-mc) was assessed. 5-mc levels were normalized to ethanol controls. Exposure to SM at 1.0 and above resulted in a significant increase of 5-mc levels. Data represent means ± SD from 3 independent biological experiments each with 3 technical replicates per group. Statistical significance is indicated by asterisks ($^*p < 0.05$ *vs.* control).

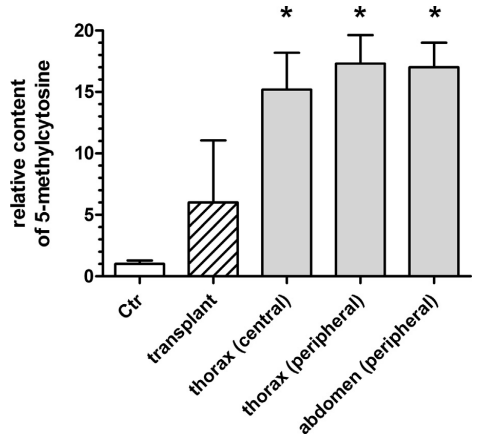

Fig. 4. DNA methylation in exposed human tissue.
Different specimens were obtained from a patient who had exposed himself to a small amount of pure sulfur mustard at the abdomen and thorax region. Due to a wound healing at the abdominal site, a skin graft was performed. The thorax area healed spontaneously. Approx. one year after exposure, a surgical revision was conducted. The transplant with adjacent potentially exposed but not excised areas (abdomen (peripheral)) and the formerly untreated thorax site (thorax (central)) and adjacent tissue (thorax (peripheral)) were removed. In addition, a control skin sample from the upper leg that was definitively not exposed to SM was obtained. Global DNA methylation (5-methylcytosine, 5-mc) was assessed in all samples. Data were normalized to control (unexposed skin) levels. Formerly exposed skin tissue (thorax) as well as potentially exposed but not primarily removed tissue (abdomen peripheral) revealed significant higher levels of 5-mc compared to control skin. The transplant, which was excised as full thickness skin, showed a distinct, but not significant increase of 5-mc levels. Bars represent means ± SD from 3 technical replicates per sample. Statistical significance is indicated by asterisks ($^*p < 0.001$ *vs.* unexposed skin).

compared to control skin. Relative 5-mc levels were determined with 15.2 ± 3.0 for the thorax central skin sample, 17.3 ± 2.3 for the peripheral thorax skin sample, 17.0 ± 2.0 for the adjacent abdominal tissue. The transplant, which was excised as full thickness skin, showed distinct, but strongly varying levels of 5-mc (6.0 ± 5.1), thus missing level of significance (Fig. 4).

4. Discussion

Prior preliminary work has proposed the involvement of epigenetic mechanisms in the pathophysiology of SM induced chronic health effects (Korkmaz et al., 2008a,b; Nourani et al., 2015). Remarkably, the histone deacetylase inhibitor Trichostatine A resulted in a significant reduction of interstitial, pulmonary edema, hemorrhage, emphysematous changes and reduction of inflammation in mechlorethamine (HN2, nitrogen mustard) induced toxic lung injury *in vivo* (Korkmaz et al., 2008b). However, underlying epigenetic pathways were not investigated in that study. It is well accepted that epigenetic pathways are involved in physiological processes like wound healing (Lewis et al., 2014) but epigenetic perturbations are also involved in the development of chronic diseases including pulmonary disorders and cancer (Ito et al., 2005; Jones and Baylin, 2002; Robertson, 2005). Moreover, endothelial dysfunction was observed in a plethora of pulmonary and cardiovascular diseases. Based on that findings and due to the fact that endothelial cells are severely affected by SM (Schmidt et al., 2009; Steinritz et al., 2010, 2011), we focused on epigenetic pathways in early endothelial cells after SM challenge in the presented study.

The term “epigenetics” refers to the temporal and spatial control of gene activity without affecting the underlying DNA nucleotide sequence. A huge number of different proteins including DNA methyltransferases, histone acetyltransferases, histone methyltransferases, SET domain proteins, histone phosphorylating proteins, proteins for histone ubiquitination, DNA/histone demethylases and histone deacetylases are known or discussed to be involved in this process. These proteins are summarized as “epigenetic modulators”. Changes of epigenetic modulators after challenge with different SM concentrations were assessed in our study using a PCR-based array that allowed the investigation of 78 different genes. Our results provide striking evidence that a single SM exposure significantly influenced epigenetic modulators 24 h after SM exposure. Remarkably, SM concentration of 0.5 and 1.0 µM caused explicit more changes in gene regulation (22 and 29 regulated genes) compared to 23.5 and 50 µM SM (10 and 10 regulated genes) (Table S1). This observation is of relevance: cells exposed to high SM concentrations (23.5 and 50 µM are both above the LC_{50}) will most probably undergo cell death within 24 h or later on. Thus, epigenetic perturbations in these cells are unlikely to persist over time and a sustainable “epigenetic risk” arising from these cells seems unlikely. However, cells exposed to low SM concentrations (0.5 and 1.0 µM are considerably below the LC_{50}) will not undergo cell death but will persist and thus epigenetic changes may have significant consequences during further lifespan.

Pronounced differences between the low dose and high dose exposure groups were observed in our experiments. SM resulted in down-regulation in the majority of affected genes in the low dose exposure group (0.5 and 1.0 µM SM) with some exceptions. An up-regulation of Dnmt1 was observed already after exposure to 0.5 µM SM and even more pronounced at 1.0 µM SM. In contrast, Dnmt1 levels were found mainly unchanged in the high-dose SM exposure group (23.5 and 50 µM SM). Dnmt1 is considered to maintain DNA methylation during replication (Jin and Robertson, 2013). This strengthens our hypothesis that particularly low SM concentrations may bear an epigenetic risk. Surprisingly, although a significant increase of global DNA methylation was observed in our experiments, an up-regulation of Dnmt3a or Dnmt3b, which are considered as responsible for DNA *de novo* methylation, was not evident. It should be noted that Dnmt1 can cause *de novo* DNA methylation itself or is at least involved in the regulation of it (Athanasiadou et al., 2010; Vertino et al., 1996). Moreover, protein

activity is not only dependent on protein expression levels but can also be modulated by post-translational modifications and protein interactions. For example, a non-covalent interaction of Dnmt1 with PARP-1 was shown that influences Dnmt1 activity and thus DNA methylation state (Caiafa et al., 2009). Remarkably, SM was found to activate PARP-1 (Debiak et al., 2009; Kehe et al., 2009a) suggesting a functional link with regard to Dnmt1 activity. A STRING analysis (Supplementary Fig. S1A) revealed that Dnmts are linked to chromatin organizing enzymes and DNA damage response genes which both may modulate DNA structure and epigenetic status. In addition, DNA methylation patterns result from both methylation and demethylation processes (Li, 2013). It can be speculated that SM induced active or passive DNA demethylation also contributed to the complex DNA methylation pattern. Recently, it has been demonstrated that Dnmts can also act as demethylases. Mammalian Dnmt1, Dnmt3a, and Dnmt3b can all convert 5-mC to cytosine (Chen et al., 2013). Moreover, histone demethylases (*i.e.*, KDM1) were shown to be involved in maintenance of global DNA methylation (Wang et al., 2009). Although KDM1 was mainly unaffected in our experiments, other KDMs (*e.g.*, KDM4c, KDM5b, KDM6b) were down-regulated in SM exposed EEC. Although unproved at the moment, it can be speculated that a link between other KDMs than KDM1 and the global DNA methylation pattern may exist. Interestingly, a functional link between KDM4c and the transcription factor Nanog was found through STRING analysis (Supplementary Fig. S1B).

Our results provide compelling evidence that epigenetic perturbations after SM exposure are of biological relevance as demonstrated by significantly increased 5-mc levels *in vivo* even one year after an accidental human exposure. Although the exact exposure dose was unknown, it can be estimated that the scenario resembles a "low-dose" exposure as the patient exposed himself to a few drops of pure SM. Increased hypermethylation, especially within tumor suppressor genes, seems to be associated with cancer development (Esteller, 2002). Although a significant increase of dermal cancer after SM exposure has not been reported, it is well known that pulmonary malignancies and cherry hemangiomas (abnormal proliferation of blood vessel) occur. We therefore recommended a cancer screening (including skin) at close intervals in addition to the regular follow up screenings for the exposed patient.

Regarding other epigenetic modulators changes in gene regulation were observed for Hat1, Hdac9, Hdac11, Kdm4c, Kdm6b, Ncoa3, Prmt8, Set1b and Whsc1. In general, the interpretation of epigenetic modulator patterns is challenging as many functions and pathways are not fully elucidated. Ncoa3 was shown to be essential for maintenance of embryonic stem cell (ESC) pluripotency (Wu et al., 2012). Down-regulation of Ncoa3 resulted in impaired differentiation potential of ESC. In our experiments, Ncoa1 and 3 were significantly down-regulated in the low-dose SM exposure group. Again, as these cell will not undergo cell death, long-term adverse effects might arise thereof. This is in line with our previous studies that exactly identified an impaired differentiation potential of murine ESC after exposure to alkylating agents (Schmidt et al., 2009; Steinritz et al., 2010).

Our results demonstrated an upregulation of Whs1c in all groups but especially in 23.5 μM exposed EEC. Here, an 11-fold increase was observable, which was the highest change in protein regulation of all investigated genes. Whsc1 has been linked with cancerogenesis (Saloura et al., 2015) and Chek2, a protein that elicits DNA repair, cell cycle arrest or apoptosis in response to DNA damage (Supplementary Fig S1C). Although 23.5 μM is above the LC_{50}, that was determined at 11.7 μM SM 24 h after exposure, and thus most of the affected cell will undergo cell death within that period or just after, it cannot be ruled out that some of these cells will survive and might promote tumor development.

Suv420h1 was shown to have direct impact on cell migration (Yokoyama et al., 2014). Our experiments revealed a distinct down-regulation of this SET domain protein in the low-dose SM exposure groups. This is in line with our recent findings showing that migration of EEC was highly affected by low concentrations of the alkylating compound chlorambucil (Steinritz et al., 2014) and with prolonged wound healing after SM exposure *in vivo* (Ghanei et al., 2010; Graham et al., 2002).

Changes of other epigenetic modulators observed in our experiments mainly addressed post-translational histone modifications (*e.g.*, acetylation, methylation, phosphorylation, ubiquitinilation, demethylation and deacetylation). Although the "histone code" has been intensively investigated during the last decade (Jenuwein and Allis, 2001; Ng and Cheung, 2015) and impressive progress has been made, the phenomena of chromatin organization and transcriptional regulation has not been entirely unraveled. Therefore, future work will focus on (i) SM induced post-translational histone modifications, (ii) the overall stability of epigenetic modulations (on both mRNA and protein levels) and (iii) their biological consequences.

Summing up, the results of our study demonstrated for the first time that SM exposure of EEC resulted in a complex regulation pattern of epigenetic modulators which was accompanied by a global increase of DNA methylation. Especially low-dose SM exposures caused comprehensive changes of the investigated epigenetic regulative genes. Examination of human skin samples one year after an accidental SM exposure revealed a significant increase of global DNA methylation, underlining the biological relevance of our findings. Our results are encouraging and future work will focus on SM induced histone modifications, the overall kinetics of epigenetic changes and therapeutic interventions *e.g.*, the use of Dnmt inhibitors. This could eventually lead to the development of new therapeutic strategies against SM induced chronic health effects as well as SM induced wound healing disorders.

Conflict of interest

The authors report no conflicts of interest.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.toxlet.2015.09.016.

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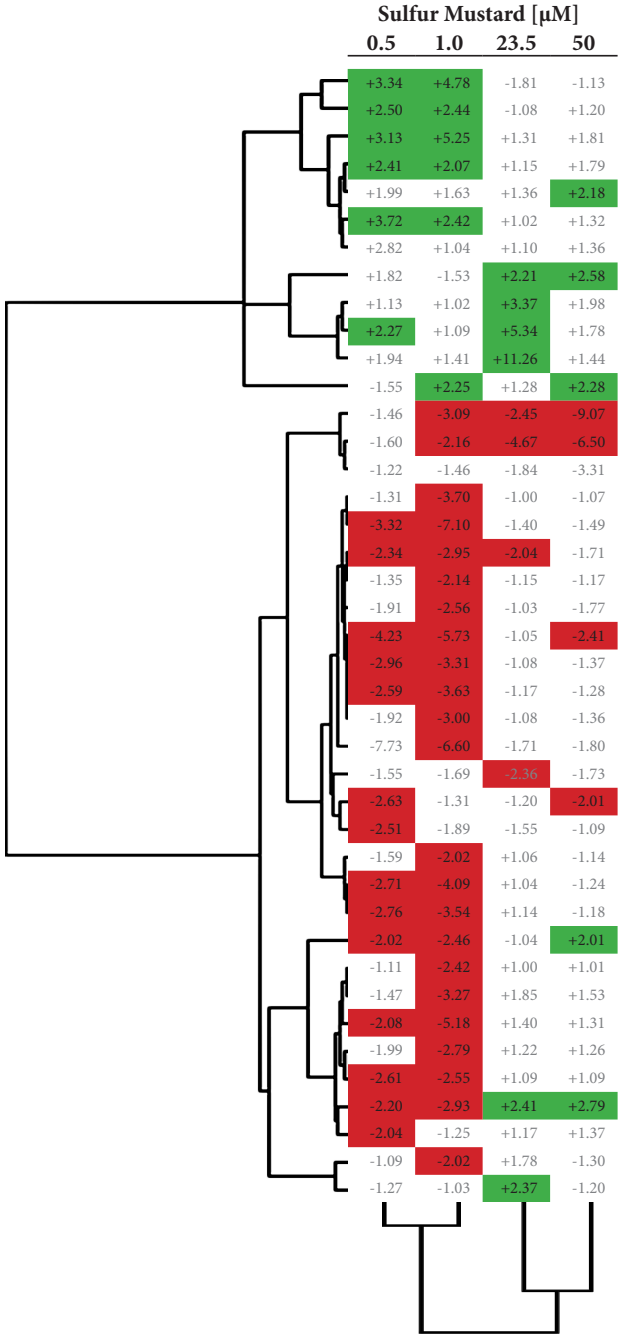
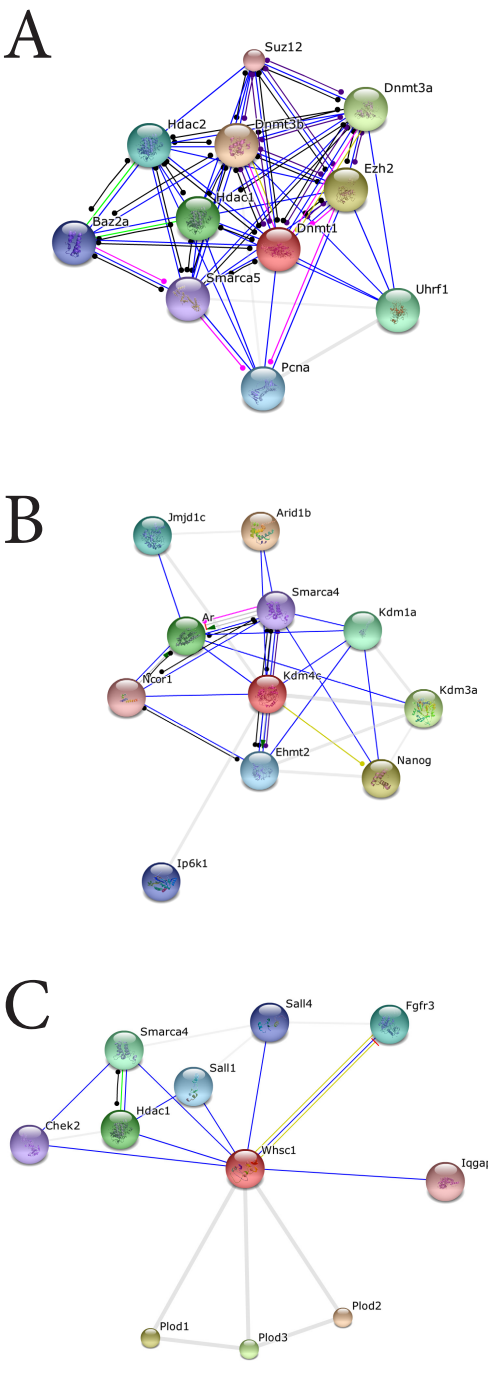
Supplementary Table 1: Data table of SM induced gene regulation in EEC



Sulfur Mustard [µM] 0.5	1.0	23.5	50	Gene	Gene Class
+3.34	+4.78	-1.81	-1.13	Dnmt1	DNA Methyltransferases
+2.50	+2.44	-1.08	+1.20	Aurkb	Histone Phosphorylation
+3.13	+5.25	+1.31	+1.81	Hat1	Histone Acetyltransferases
+2.41	+2.07	+1.15	+1.79	Esco2	Histone Acetyltransferases
+1.99	+1.63	+1.36	+2.18	Aurka	Histone Phosphorylation
+3.72	+2.42	+1.02	+1.32	Prmt7	Histone Methyltransferases
+2.82	+1.04	+1.10	+1.36	Aurkc	Histone Phosphorylation
+1.82	-1.53	+2.21	+2.58	Smyd1	Histone Methyltransferases
+1.13	+1.02	+3.37	+1.98	Setd8	SET Domain Proteins
+2.27	+1.09	+5.34	+1.78	Prmt8	Histone Methyltransferases
+1.94	+1.41	+11.26	+1.44	Whsc1	SET Domain Proteins
-1.55	+2.25	+1.28	+2.28	Setd6	SET Domain Proteins
-1.46	-3.09	-2.45	-9.07	Dnmt3a	DNA Methyltransferases
-1.60	-2.16	-4.67	-6.50	Kdm4c	DNA / Histone Demethylases
-1.22	-1.46	-1.84	-3.31	Smyd3	Histone Methyltransferases
-1.31	-3.70	-1.00	-1.07	Kdm5c	DNA / Histone Demethylases
-3.32	-7.10	-1.40	-1.49	Kdm6b	DNA / Histone Demethylases
-2.34	-2.95	-2.04	-1.71	Ncoa3	Histone Acetyltransferases
-1.35	-2.14	-1.15	-1.17	Cdyl	Histone Acetyltransferases
-1.91	-2.56	-1.03	-1.77	Hdac4	Histone Deacetylases
-4.23	-5.73	-1.05	-2.41	Ncoa1	Histone Acetyltransferases
-2.96	-3.31	-1.08	-1.37	Kdm5b	DNA / Histone Demethylases
-2.59	-3.63	-1.17	-1.28	Suv420h1	SET Domain Proteins
-1.92	-3.00	-1.08	-1.36	Setd5	SET Domain Proteins
-7.73	-6.60	-1.71	-1.80	Hdac9	Histone Deacetylases
-1.55	-1.69	-2.36	-1.73	Dnmt3b	DNA Methyltransferases
-2.63	-1.31	-1.20	-2.01	Kat2b	Histone Acetyltransferases
-2.51	-1.89	-1.55	-1.09	Ncoa6	Histone Acetyltransferases
-1.59	-2.02	+1.06	-1.14	Hdac8	Histone Deacetylases
-2.71	-4.09	+1.04	-1.24	Setd1b	SET Domain Proteins
-2.76	-3.54	+1.14	-1.18	Nsd1	SET Domain Proteins
-2.02	-2.46	-1.04	+2.01	Setd1a	SET Domain Proteins
-1.11	-2.42	+1.00	+1.01	Dot1l	Histone Methyltransferases
-1.47	-3.27	+1.85	+1.53	Kdm4a	DNA / Histone Demethylases
-2.08	-5.18	+1.40	+1.31	Hdac11	Histone Deacetylases
-1.99	-2.79	+1.22	+1.26	Setd7	SET Domain Proteins
-2.61	-2.55	+1.09	+1.09	Mysm1	Histone Ubiquitination
-2.20	-2.93	+2.41	+2.79	Usp22	Histone Ubiquitination
-2.04	-1.25	+1.17	+1.37	Ube2b	Histone Ubiquitination
-1.09	-2.02	+1.78	-1.30	Ciita	Histone Acetyltransferases
-1.27	-1.03	+2.37	-1.20	Rnf2	Histone Ubiquitination

Fold-change values of genes that were regulated >2 fold in at least one group. Up-regulated genes are illustrated in green and down-regulated genes in red. Other than in the heat map (Fig. 2A) colors do not indicate gradations in gene regulation. Hierarchical clustering with distance measures based on the Pearson correlation is indicated by a dendogram.

<u>Supplementary Figure 1:</u> Protein Association Network Analysis using STRING



A protein association network analysis was conducted for selected epigenetic modulators using the STRING database (www.string-db.com).

(A) Dnmt1, Dnmt3a or Dnmt3b were used as input variables. The result indicated a direct interaction with histon modifying proteins (e.g. Hdac1, Hdac2). In addition, other proteins involved in the organization of chromatin structure (e.g. Baz2a, Smarca5, Suz12) were also found to interact with Dnmts. Moreover, DNA damage response proteins (e.g. Pcna) were associated within the functional protein network.

(B) Kdm4c as input variable revealed that expression of the transcription factor Nanog, which is responsible for self-renewal of undifferentiated embryonic stem cells while loss of Nanog function causes differentiation, is discussed to depend on Kdm4c expression.

(C) Whsc1, the protein with highest change in regulation of all investigated genes, was found to be involved in collagen biosynthesis (Plod1-3) but also associated with Chek2, a protein that elicits DNA repair, cell cycle arrest or apoptosis in response to DNA damage.

Color legend (for details see www.string-db.com):
green = activation
red = inhibiton
blue = binding
purple = post-translational modification
yellow = expression
black = reaction

3.2 Sulfur Mustard-Induced Epigenetic Modifications Over Time - A Pilot Study

Simons, Thilo; Steinritz, Dirk; Bölck, Birgit; Schmidt, Annette; Popp, Tanja; Thiermann, Horst; Gudermann, Thomas; Bloch, Wilhelm; Kehe, Kai, Toxicology Letters, 16 of November 2017, Vol. 293, pp. 45-50

Additionally to short-term exposure of early endothelial cells to SM (section 3.1), we were able to investigate epigenetic changes over time. In particular, we analyzed the effects of SM on the global DNA methylation state, histone acetylation and histone di-methylation at exemplary histone sites (acetyl/di-methyl H3-K9, acetyl/di-methyl H3-K27, di-methyl H3-K36, acetyl H4-K8) 24 hours after exposure as well as after additional 2 and 4 cell passages. Also the skin samples of the accidentally exposed patient (section 3.1) were examined on the same terms and compared to the *in vitro* tests. Applied investigation techniques used to detect epigenetic modifications included ELISA based assay, immunohistochemical procedures, immunofluorescence labelling as well as confocal microscopy of tissue sections. Subsequently we conducted a statistical analysis. After an initial decrease of 5-mc levels as a marker for the DNA methylation state in the low exposure groups (0.5 μM and 1.0 μM) we found that 5-mc levels increased after 4 cell passages as well as in the obtained skin samples. Histone modifications appeared not to be consistent presenting strong variations over time and dose. Acetylation tended to return to control level over time (H3-K27), decrease (H3-K9) or increase (H4-K8). Also histone di-methylation (H3-K9, H3-K27, H3-K36) appeared to return to control level or to decrease over time evincing strong variations and depending onto the cell type (EECs or skin cells). The key findings can be summarized as follows: (I) SM exposure caused a significant increase of 5-mc levels over time in EECs as well as in the skin samples, and (II) occurrence and persistence of histone modifications remained statistically unsignificant.

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Full Length Article

Sulfur mustard-induced epigenetic modifications over time – a pilot study

Thilo Simons[a], Dirk Steinritz[a,b], Birgit Bölck[c], Annette Schmidt[a,d], Tanja Popp[a,b], Horst Thiermann[a], Thomas Gudermann[b], Wilhelm Bloch[c], Kai Kehe[a,e,*]

[a] Bundeswehr Institute of Pharmacology and Toxicology, 80937, Munich, Germany
[b] Walther-Straub-Institute of Pharmacology and Toxicology, Ludwig-Maximilians-Universität München, 80336 Munich, Germany
[c] Department of Molecular and Cellular Sports Medicine, German Sports University, 50933, Cologne, Germany
[d] Bundeswehr University Munich, Faculty of Human Sciences, 85577, Neubiberg, Germany
[e] Bundeswehr Medical Academy, Dept. Medical CBRN Defense, 80937, Munich, Germany

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ABSTRACT

The chemical warfare agent sulfur mustard (SM) can cause long-term health effects that may occur even years after a single exposure. The underlying pathophysiology is unknown, but epigenetic mechanisms are discussed as feasible explanation. "Epigenetics" depicts regulation of gene function without affecting the DNA sequence itself. DNA-methylation and covalent histone modifications (methylation or acetylation) are regarded as important processes. In the present *in vitro* study using early endothelial cells (EEC), we analyzed SM-induced DNA methylation over time and compared results to an *in vivo* skin sample that was obtained approx. one year after an accidental SM exposure. EEC were exposed to low SM concentrations (0.5 and 1.0 µM). DNA methylation and histone acetylation (H3-K9, H3-K27, H4-K8) or histone di-methylation (H3-K9, H3-K27, H3-K36) were investigated 24 h after exposure, and after 2 or 4 additional cell passages. The human skin sample was assessed in parallel. SM had only some minor effects on histone modifications. However, a significant and pronounced increase of DNA methylation was detected in the late cell passages as well as in the skin sample. Our results indicate that SM does indeed cause epigenetic modifications that appear to persist over time.

1. Introduction

Exposure to the chemical warfare agent sulfur mustard (SM; CAS-Nr. 505-60-2) causes both acute and long-term health effects (Ghabili et al., 2010). The underlying pathophysiology is still not understood. Acute effects are supposed to be caused by DNA alkylation and the consequences thereof. DNA adducts, however, are recognized by cellular DNA damage response elements (Matijasevic et al., 2001) and cells either initiate DNA repair processes or may activate cell death programs in case of very severe DNA affections (Ruff and Dillman, 2007). In SM exposure cases presenting mild symptoms, a complete recovery is usually observed (Steinritz et al., 2016b). However, some patients develop long-term and delayed clinical symptoms, even after a single exposure (Balali-Mood et al., 2005; Ghanei and Harandi, 2007; Firooz et al., 2011; Kehe et al., 2016). The reasons for those late effects remain obscure and a meaningful pathophysiological explanation for delayed SM-induced chronic health effects is still lacking. However, recent studies have proposed epigenetic alterations or an imbalance of the existing epigenetic pattern as possible cause (Korkmaz et al., 2008; Imani et al., 2015; Steinritz et al., 2016a). "Epigenetics" describe functionally relevant changes to the genome without direct mutational modifications in the DNA sequence (Weinhold, 2006; Sharma et al., 2010; Conaway, 2012). Epigenetic modifications can be grouped into different categories: i) changes of the DNA methylation status, ii) histone modifications, and iii) influence on protein synthesis by noncoding RNAs like miRNA or long non-coding RNA (lncRNA) (Handy et al., 2011; Du et al., 2015; Imani et al., 2015). In a previous *in vitro* study, we have already demonstrated that exposure to SM, especially at very low concentrations at which cell death is negligible, affected the expression of epigenetic modulators and the global DNA methylation status of early endothelial cells significantly (Bloch et al., 2016, 2017; Steinritz et al., 2016a). In the same study, we had the chance to investigate the global DNA methylation status of a human sample after an accidental SM exposure. Here, a significant increase of 5-methylcytosine (5-mC) was detected in the previous SM-exposed skin areas compared to non-exposed skin samples indicating that SM indeed

Abbreviations: 5-mC, 5-methylcytosine; AU, arbitrary unit; DMEM, Dulbecco's Modified Eagle medium; FCS, fetal calf serum; EEC, early endothelial cells; EtOH, ethanol; H3-K9, histone 3 lysine 9; H3-K27, histone 3 lysine 27; H3-K36, histone 3 lysine 36; H4-K8, histone 4 lysine 8; HRP, horseradish peroxidase; MEM, minimum essential medium; NMR, nuclear magnetic resonance; P, cell passage; SM, sulfur mustard

* Corresponding author at: Bundeswehr Medical Academy, Dept. Medical CBRN Defense, Munich, 80937, Germany.
E-mail address: kai.kehe@lrz.uni-muenchen.de (K. Kehe).

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influenced DNA methylation and that these effects seem to persist substantially (Steinritz et al., 2016a). Nevertheless, little is known about the stability of epigenetic modifications over time. The persistence of epigenetic differences – even over decades – associated with prenatal exposure to famine is one example that indicates a high stability of such modifications (Heijmans et al., 2008; Talens et al., 2010), while a longitudinal study of epigenetic variation in twins (Wong et al., 2010) describes the opposite result. It is assumed that SM does induce epigenetic modifications. However, this is difficult to investigate in vivo because the individual epigenetic status before exposure is unknown in the majority of cases and makes thus interpretation of epigenetic modifications after exposure challenging. Thus, we have initiated another *in vitro* study to investigate SM-induced epigenetic changes (DNA methylation and histone modifications) over time, using a well-established cell culture system with early endothelial cells, and compared our results to a skin samples of a SM-exposure case.

2. Materials and methods

2.1. Cell culture

Early endothelial cells (EEC) were obtained from differentiated murine embryoid bodies as described previously (Schmidt et al., 2004). PECAM-1 positive fraction of cells (in the following named "MACS cells") were cultured on gelatine-coated dishes in DMEM (Dulbecco's Modified Eagle medium) supplemented with 15% heat-inactivated fetal calf serum (FCS), 50 U/ml penicillin, 50 U/ml Streptomycin, 200 mM L-glutamine, 100 mM b-mercaptoethanol, and 1% MEM (non-essential amino acids (GIBCO-BRL, Gaithersburg, USA)). After 2–3 weeks, the endothelial cells were passaged for the first time. Subsequently, cells were split before reaching confluency and were used up to passage 8. Cells were handled under sterile conditions and cultivated with 5% CO_2 at 37 °C and 95% humidity.

2.2. In vivo material

SM-exposed human skin as well as control skin was obtained from a patient that accidently exposed himself to SM and required skin debridement and a split-skin graft (Steinritz et al., 2016a). A subsequent surgical procedure was conducted 1 year after the initial event. Skin samples of the formerly SM-exposed area of the upper thorax, and control skin from a definite non-exposed area from the thigh were collected. The patient's consent and approval of the ethic committee was obtained in advance.

2.3. SM exposure

SM was made available by the German Ministry of Defense. A purity of at least 99% was confirmed by NMR analysis. SM was diluted in EtOH resulting in a 400 µM stock solution. The stock solution was further diluted in DMEM directly before exposure and added to the cells resulting in SM doses of 0.5 and 1.0 µM in accordance to our previous study (Steinritz et al., 2016a). Controls were treated with EtOH (0.25 Vol-%).

2.4. Investigation of global DNA methylation (5-mC)

Global DNA methylation was assessed by determination of 5-methylcytosine (5-mC) using an ELISA-based assay (5-mC kit; Zymo Research, California, USA) following the manufacturer's instructions. Every DNA sample was diluted in the provided coating buffer to a final concentration of 1 ng/ml. Denaturation of the DNA was done at 98 °C for 5 min. Denaturated DNA was immediately transferred to ice for 10 min, then transferred to a 96-well plate. and the plate was incubated for 1 h at 37 °C. After three washing steps with the ELISA buffer, the antibody mix was added. Samples were incubated for another 1 h at 37 °C. Plates were then washed again 3-times with ELISA buffer. After developing the signal with HRP-developer solution, absorption was measured at 405 nm using a plate reader (Multiskan FC, Thermo Scientific, USA). Values were normalized to the 5-mC levels of the EtOH controls or to the levels of the control skin. The 5-mC ratios were calculated in relation to the absorbance, detected using an ELISA plate reader, of the standard curve obtained from the positive controls at 405 nm wavelength.

2.5. Detection of histone-modifications

2.5.1. Immunocytochemistry

All cells were fixed after treatment ± intervention with 4 % paraformaldehyde in 0.1 M PBS for 25 min. and washed three times with 0.1 M PBS. The permeabilization of cells is an important point to facilitate the antibody binding to inner cell proteins. Therefore, the cells were incubated 10 min with 0.25 % Triton-X 100 and 0.5 M NH_4Cl in 0.05 M Tris-Buffered-Saline (TBS). The detergent Triton-X 100 permeabilizes the cell membrane while the NH_4Cl reacts with free aldehyde groups to prevent an unspecific binding of the antibodies, followed by rinsing with TBS (3 x 10 min). To prevent unspecific bindings cells were incubated with 2 % bovine serum albumin (BSA) in 0.05 M TBS (1 h at RT). Primary monoclonal antibodies against acetylated lysine residues of histone 3, lysine 9 (Ac-H3-K9), Ac-H3-K27, Ac-H4-K8 or against dimethylated lysine residues of histone 3, lysine 9 (DM-H3-K9) as well as DM-H3-K27 or DM-H3-K36 (all antibodies were from Cell Signaling and diluted 1:1000) were used. Cells and tissue samples were incubated at 4 °C over night. The following day cells were rinsed with TBS and then incubated with the corresponding biotinylated secondary antibody, either goat anti-rabbit IgG (1:500 in 0.05 M TBS) for 1 h followed by a streptavidin Alexa555 (1:500 in 0.05 M TBS) (life technologies, USA) for 1 h. Finally, staining with DRAQ5 in 0.1 M PBS was done for 10 min to visualize the nucleus.

2.5.2. Immunofluorescence labelling and confocal microscopy of tissue sections

The tissue slices were incubated with rabbit anti-AC H3-K27 (1:1000) for 24 h at 4° C. The sections were incubated with biotinylated goat anti rabbit IgG (1:500) and with the Streptavidin Alexa 555 (1:500) for 1 h at RT, respectively. Staining of nuclear DNA staining was done using DRAQ5 (1:2000). Control experiments were performed in separate incubations by omission of the primary or secondary antibodies. Two color fluorescent images were acquired on an LSM 510 META confocal microscope (Carl Zeiss, Oberkochen, Germany). The 543 nm excitation beam and 560–613 nm band-pass emission filter were used to selectively view the red fluorochrome (for the identification of Histone modification). The 633 nm excitation beam and 649–702 nm band-pass emission filter were used to selectively view the far-red fluorochrome (for the identification of DRAQ5). The measurement of the staining intensity was performed by using the Image J Software (National Institutes of Health, Bethesda, Maryland, USA). The blue colored nucleus was manually selected and then both channels 633 nm (blue; DRAQ5) and 543 nm (Red; Alexa 555) were measured. In the program, the signal strength and the area, that were averaged over the whole nucleus, for each channel and nucleus were stored in a database. For every condition (control, Treatment) 50 scans were performed. Dermal tissue slices were stained according to the same protocol. Epidermal cell layers were categorized into basal cells (B), intermediate cells (I) and apical cells (A) and were evaluated independently.

2.6. Statistics

Statistically significant differences between the means of respective groups were determined by one-way ANOVA and Tukey-Kramer correction for multiple testing using GraphPad Prism v7.03 (GraphPad

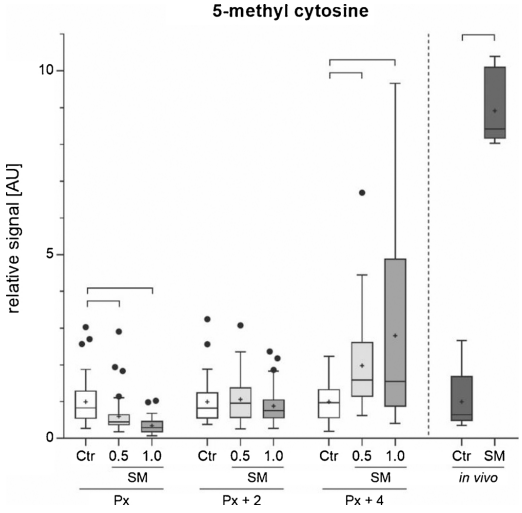


Fig. 1. Long-term global DNA methylation after SM exposure.
Early endothelial cells were exposed to sulfur mustard (SM) (0.5 or 1.0 μM) or sham-treated with ethanol (Ctr). After 24 h (Px), global 5-mC levels were assessed by ELISA. Cells were cultivated for two (Px + 2) or four (Px + 4) additional cell passages. Levels of 5-mC were assessed at each passage (Px, Px + 2, Px + 4). Human skin samples, obtained approx. one year after an accidental SM exposure, were investigated in parallel. Results from three independent experiments (n = 3) with a total number of 50 cells per group and time point were analyzed and are visualized by Tukey boxplots (median, lower and upper quartile, whiskers at 1.5 IQR, outliers as individual points). Horizontal brackets encompass all groups with significant differences ($p \leq 0.01$) compared to the ethanol control group or the control *in vivo* skin sample

Software, La Jolla, USA). P-values < 0.01 were regarded as significant. Experiments were repeated at least three times (n = 3) for the *in vitro* experiments, while only a single *in vivo* sample (n = 1) derived from the only patient was available. Specimens from the thorax region, which was initially exposed to SM, and a control region were divided into three parts that were investigated separately, resulting in three technical replicates. In-depth statistical analysis was not conducted for the *in vivo* samples.

3. Results

3.1. Effects of SM on the global DNA methylation state

Changes of global DNA methylation state was assessed by determination of overall 5-mC levels by ELISA technique. After exposure of EEC (initial cell passage was defined at "Px" at the beginning of the study) with SM, 5-mC levels decreased significantly within 24 h in both SM groups (Fig. 1, $Px_{0.5\ \mu M} = 0.604 \pm 0.484$, $Px_{1\ \mu M} = 0.347 \pm 0.217$). After cultivation for another two passages, global 5-mC levels returned to control levels in both SM exposure groups (Fig. 1, $Px + 2_{0.5\ \mu M} = 1.061 \pm 0.598$, $Px + 2_{1\ \mu M} = 0.884 \pm 0.466$). Remarkably, after another two passages, relative 5-mC levels significantly increased again in both SM groups, but especially in the 1.0 μM SM group (Fig. 1, $Px + 4_{1\ \mu M} = 2.8 \pm 2.552$). The variation in this group was more pronounced than in the 0.5 μM SM group (Fig. 1, $Px + 4_{0.5\ \mu M} = 1.985 \pm 1.186$).

In the human *in vivo* samples, that were obtained approx. one year after an accidental SM exposure, 5-mC levels of the unexposed skin were comparable to the *in vitro* control groups. However, 5-mC levels increased significantly and pronounced in the formerly SM-exposed skin area (Fig. 1, $in\ vivo_{SM} = 8.916 \pm 1.021$).

3.2. SM-induced histone modifications

Acetylation and di-methylation at three exemplary histone-sites were investigated by immunocytochemistry. In general, all investigated post-translational modifications exhibited some changes. However, changes were not consistent and showed variations regarding time and SM exposure concentrations. A clear dose dependency could not be observed in the *in vitro* samples.

3.2.1. Histone acetylation

Histone acetylation that is linked to gene activation was investigated by immunohistochemical staining of acetyl H3-K9 (Fig. 2A), acetyl H3-K27 (Fig. 2B), and acetyl H4-K8 (Fig. 2C).

Exposure of EEC to SM resulted in a significant increase of acetyl H3-K9 within 24 h after exposure with a pronounced increase in the 0.5 μM SM group ($Px_{0.5\ \mu M} = 2.119 \pm 0.766$). With ongoing cell culturing at Px + 2 and Px + 4, acetyl H3-K9 levels returned almost to control levels. Some statistical differences were detected; however, differences were small. At Px + 4, no differences were found between control cells and cells formerly exposed to 1.0 μM SM. Basal acetyl H3-K9 levels were homogenously distributed in control skin. In the skin areas, that were exposed to an unknown amount of SM approx. one year ago, a weak decrease in acetyl H3-K9 levels was determined in the basal, intermediate and apical cell layers (B = 0.582 ± 0.527, I = 0.572 ± 0.460 and A = 0.556 ± 0.359).

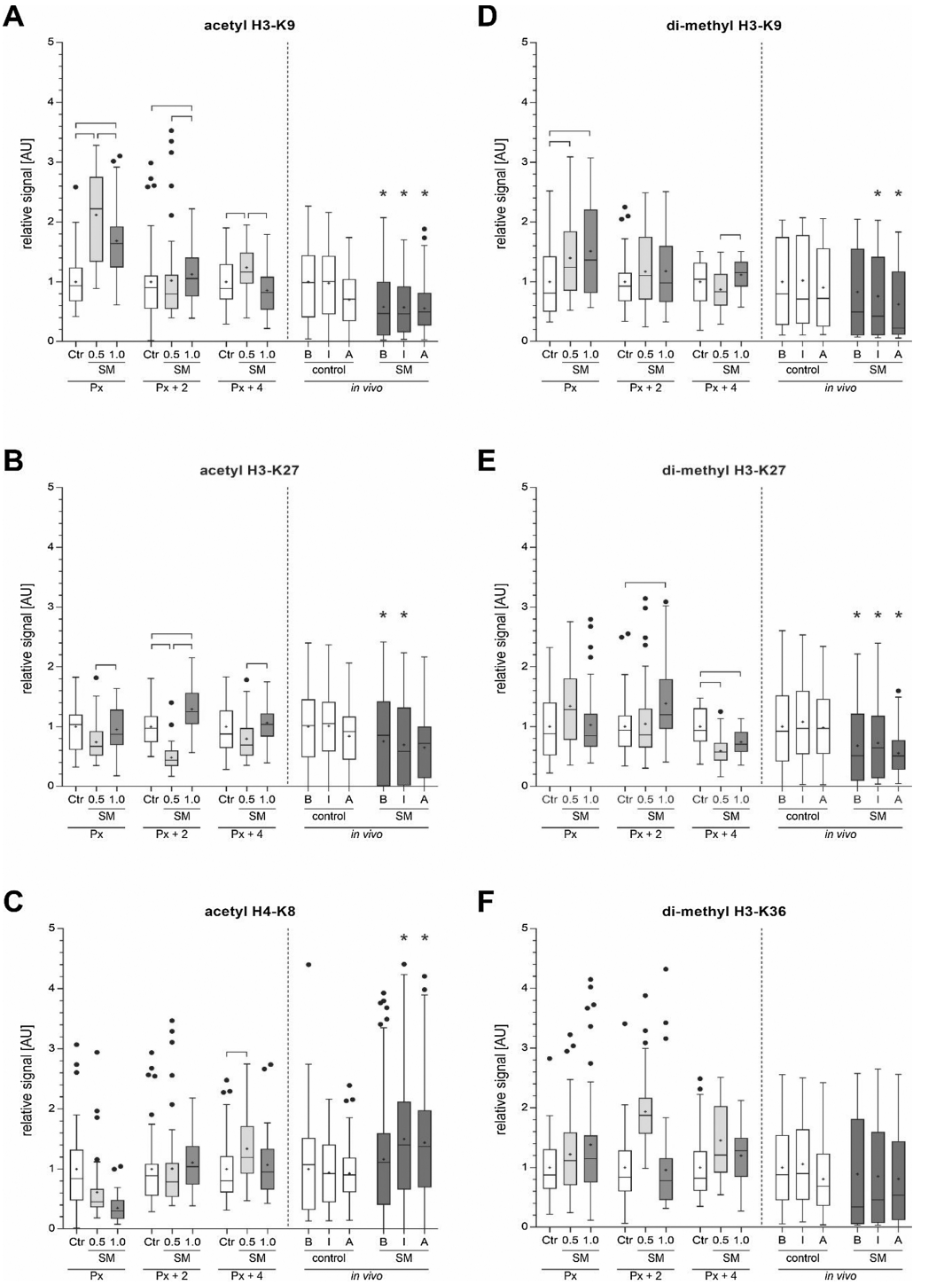
Regarding acetyl H3-K27, only very minor changes were observed. Statistical differences between the SM groups and the control cells were not detected 24 h after exposure (Px). In Px + 2 some differences were evident, but a dose-dependency could not be observed. Cells, formerly exposed to 0.5 μM SM exhibited a decrease of acetyl H3-K27 levels ($Px + 2_{0.5\ \mu M} = 0.483 \pm 0.219$), while exposure to 1.0 μM resulted in an increase ($Px + 2_{1\ \mu M} = 1.292 \pm 0.385$). After another two cell passages (Px + 4), there was again no difference between the SM groups and control cells. In the 0.5 μM SM group, acetyl H3-K27 levels increased compared to Px + 2 but were still significantly below those levels in the 1.0 μM SM group. In the control skin, basal acetyl H3-K27 levels were homogenously distributed in all cell layers, in the same manner as the acetyl H3-K9 distribution pattern. In the SM-exposed skin, levels of H3-K27 were found significantly decreased in basal, intermediate and apical cell layers (B = 0.756 ± 0.743, I = 0.697 ± 0.672 and A = 0.653 ± 0.493).

Stainings of acetyl H4-K8 revealed distinct variations regarding the distribution of the raw values in all experimental groups. Due to this effect, no relevant statistical differences were found in the *in vitro* experiments. By trend, SM decreased acetyl H4-K8 levels 24 h after exposure in Px ($Px_{0.5\ \mu M} = 0.612 \pm 0.49$, $Px_{1\ \mu M} = 0.351 \pm 0.219$). The results suggest a dose-response relationship. Levels of acetyl H4-K8 returned to control levels in Px + 2 and Px + 4. Basal levels of acetyl H4-K8 were again homogenously distributed in the non-exposed control skin. In the SM-exposed skin, acetyl H4-K8 staining was strikingly enhanced in the intermediate and apical cell layers (I = 1.499 ± 1.059 and A = 1.439 ± 0.958). Here, a significant difference to the basal cell layer of the control skin was observed. In the basal layer of the exposed skin, an enhanced staining was noticed by trend, however, a significant difference could not be identified (B = 1.161 ± 0.843).

3.2.2. Histone di-methylation

Histone methylation that is linked to gene silencing was investigated by immunohistochemical staining of di-methyl H3-K9 (Fig. 2D), di-methyl H3-K27 (Fig. 2E), and di-methyl H4-K36 (Fig. 2F).

SM exposure resulted in a significant increase of di-methyl H3-K9 levels in EEC 24 h after exposure in Px ($Px_{0.5\ \mu M} = 1.397 \pm 0.675$, $Px_{1\ \mu M} = 1.511 \pm 0.732$). A dose-response relationship was noticed by trend, however, at a p-value of 0.01 a statistical difference between 0.5 and 1.0 μM SM was not found. After additional two cell passages (Px + 2), no variation in the di-methyl H3-K9 levels were identified.



(caption on next page)

Fig. 2. Long-term SM-induced histone modifications.
Post-translational histone modifications (acetylation or di-methylations) were investigated by immunocytochemistry either *in vitro* or *in vivo*. Early endothelial cells were exposed to sulfur mustard (SM) (0.5 or 1.0 μM) or sham-treated with ethanol (Ctr). After 24 h (Px), cells were fixed, stained and investigated using a laser-scanning-microscope. Cells were cultivated for two additional cell passages (Px + 2) and two further passages (Px + 4). Histone modifications (acetyl H3-K9 (A), acetyl H3-K27 (B), acetyl H4-K8 (C), di-methyl H3-K9 (D), di-methyl H3-K27 (E), and di-methyl H3-K36 (F)) were assessed at each passage (Px, Px + 2, Px + 4). Human skin samples, obtained approx. one year after an accidental SM exposure, were investigated in parallel. Levels of histone modifications were assessed in the epidermal basal cells ("B"), in intermediate cell layer ("I") or in the apical cells ("A"). Results from three independent experiments (n = 3) with a total number of 50 cells per group and time point were analyzed and are visualized by Tukey boxplots (median, lower and upper quartile, whiskers at 1.5 IQR, outliers as individual points). Horizontal brackets encompass all groups with significant differences ($p \leq 0.01$) compared to the ethanol control group or the control *in vivo* skin sample.

Another two cell passages (Px + 4) had no additional effect, however, a significant difference between the 0.5 and 1.0 μM SM was found ($Px_{0.5\ \mu M} = 0.87 \pm 0.354$, $Px_{1\ \mu M} = 1.116 \pm 0.268$). Basal levels of di-methyl H3-K9 were homogenously distributed in the non-exposed control skin. In the SM-exposed skin, basal cells revealed no difference compared to basal cells of control skin, whereas intermediate and apical cells exhibited lower levels (B = 0.830 ± 0.754, I = 0.758 ± 0.696 and A = 0.624 ± 0.579).

A comparable pattern was found for di-methyl H3-K27. In Px, no differences were found between control cells and SM-exposed cells ($Px_{0.5\ \mu M} = 1.341 \pm 0.645$). In Px + 2, only 1.0 μM exposed cells exhibited some increase of di-methyl H3-K27 levels ($Px + 2_{1\ \mu M} = 1.385 \pm 0.627$). However, di-methyl H3-K27 levels significantly decreased in Px + 4 in both SM exposure groups compared to control cells ($Px + 4_{0.5\ \mu M} = 0.594 \pm 0.232$, $Px + 4_{1\ \mu M} = 0.741 \pm 0.214$). Again, basal levels of di-methyl H3-K27 were homogenously distributed in the non-exposed control skin. Also, comparable to the findings regarding di-methyl H3-K9, SM-exposed skin showed a decrease of di-methyl H3-K27 that was found most dominant in the apical cell layer (B = 0.679 ± 0.624, I = 0.724 ± 0.56 and A = 0.554 ± 0.355).

Results regarding di-methyl H3-K36 exhibited no statistical differences in all *in vitro* and *in vivo* experiments. However, variations of the relative signal values were found to decrease over time especially in the SM exposure groups. While the distribution of di-methyl H3-K36 was homogenous in all layers of the control skin, levels were decreased by trend in all cell layers of formerly SM-exposed skin (B = 0.89 ± 0.922, I = 0.851 ± 0.861 and A = 0.810 ± 0.739).

4. Discussion

Epigenetic modifications are proposed to cause various biological phenomena and long-term health effects that are difficult to explain otherwise. A good example are SM-induced delayed or long-term health effects that can still occur years after a single exposure (Balali-Mood et al., 2005; Kehe et al., 2016). While acute health effects seem to rely on DNA alkylation and the biological consequences thereof, SM-induced epigenetic DNA modifications are discussed to be responsible for long-term effects (Korkmaz et al., 2008; Steinritz et al., 2016a). In general, the concept of epigenetics describes affection of gene expression independent of DNA sequence transformation (Meyer and Widom, 2005; Matouk and Marsden, 2008). It is well accepted that epigenetic information is inherited in plants (Hauser et al., 2011). If this holds also true in mammals is debated controversially (Heard and Martienssen, 2014; Vaiserman, 2015; van Otterdijk and Michels, 2016). There is, however, some good evidence that the individual epigenetic information, that has been imprinted by e.g. environmental events, does persist in the individuum (Heijmans et al., 2008; Baccarelli and Bollati, 2009; Hashimoto et al., 2010). In this context, it is of utmost importance to understand that epigenetic modifications are not in fact "static" but may be highly dynamic over time (Reik, 2007; Bjornsson et al., 2008). In a previous study (Steinritz et al., 2016a), we have successfully demonstrated that exposure to alkylating compounds affects epigenetic modulators and 5-mC levels early after exposure. Results, that were obtained from a human *in vivo* sample, also suggested a certain stability of these modifications over time. Because *in vivo* material is rare or even not available, a definitive answer to the hypothesis that SM-induced epigenetic modifications do persist could not be given. Therefore, we conducted the presented study that analyzed epigenetic modifications *in vitro* over time and cell generations, i.e. over a period of six cell passages. Moreover, we compared the *in vitro* results to the *in vivo* data, again, obtained from the only available human sample in our lab.

While DNA methylation is accepted to be linked to gene silencing, the role of histones and post-translational histone modifications with regard to gene activity is less clear. It was assumed that histones are mandatory for chromosome modelling, recent findings indicate that the gross structure of chromosomes will even form without histones (Kakui and Uhlmann, 2017). This may lead to the hypothesis that histones hold a function that goes beyond maintaining chromatin structure, namely regulating gene transcription. In general, histone methylation to various degree is thought to either inhibit or activate transcription while histone acetylation may promote transcription (Kuo and Allis, 1998; Jenuwein and Allis, 2001). Moreover, the balance between acetylation and methylation is considered as crucial for gene accessibility (Ohzeki et al., 2012). In addition, a precise link between histone modification and an exact gene regulation has not been established so far. The biological relevance of a certain histone modification is therefore hardly to predict. We have chosen to investigate histone H3 and H4 modifications in our study, as histone H3 is discussed to be the most modified histone in the epigenetic context and histone H4 is an important protein in the structure and function of chromatin. Modifications of other histones were not in the focus of the presented study. However, we have performed some additional experiments including additional histone modifications. All investigated histones showed the same dynamic in our experiments, thus we do assume that SM-induced post-translational histone modifications do not seem to play a major role with regard to SM-induced epigenetic long-term effects. This hypothesis is underlined by the *in vivo* results that do not exhibit dramatical changes in SM-exposed skin compared to control skin. At this point, our results can be interpreted in terms of that DNA methylation seems to be a key event after SM exposure while histone modifications are not.

We must admit that our study exhibits a relevant shortcoming, that must be addressed: we investigated EEC in the *in vitro* experiments and compared the results with epidermal keratinocytes in the *in vivo* samples. One may argue that this is to compare apples and oranges. The reason for that approach is, however, simple. The *in vitro* study was conducted before we got hands on the human samples and evaluation of endothelial cells in the human sample would have been highly challenging, as these cells are not highly abundant in histochemical sections. Nevertheless, we assume that our findings hold true for the general situation after SM exposures.

Future experiments are currently planned that will use *in vivo* models over a prolonged time to assess SM-induced epigenetic modifications. Moreover, due to the scientific progress it will be possible to use highly sensitive and specific mass spectrometry methods to characterize epigenetic DNA modifications (Bareyt and Carell, 2008) and to identify possible epigenetic intermediates (Su et al., 2016) in more detail.

In summary, our data suggest that SM does induce stable epigenetic modifications. These are predominantly represented by DNA methylation while histone modifications seem to play a minor role only. *In vivo* study should be conducted to validate the results of our pilot study and

to provide more insight into SM-induced epigenetic modifications that may be responsible for SM long-term health effects.

Conflict of interest

The authors report no conflicts of interest.

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4 Summary and Outlook

4.1 Approach and Results

The goal of the research conducted was to examine the action of epigenetic mechanisms and modifications in early endothelial cells after poisoning with SM, which contribute to the acute and long-term effects patients have to suffer from. Of particular interest were affected cells that survived the primary contact with the agent and persisted in an altered state. In contrast to cells that were affected by a high dose of SM it is assumed that low-dose affected cells would not immediately go through necrotic or apoptotic processes and that epigenetic cellular abnormalities therefore may occur after subsequent cell divisions [133]. These viable but potentially epigenetically modified cells might contribute to the observed long-term complications of SM intoxication. Epigenetic alterations were identified by measuring changes of several representative marker molecules and genes. Short-term and long-term changes were examined separately. Applied analytic techniques included the usage of PCR array, genomic DNA extraction kit, ELISA-based assay as well as immunofluorescent techniques. An exemplaric figure of cell nucleus analysis is pictured in figure 4.1.

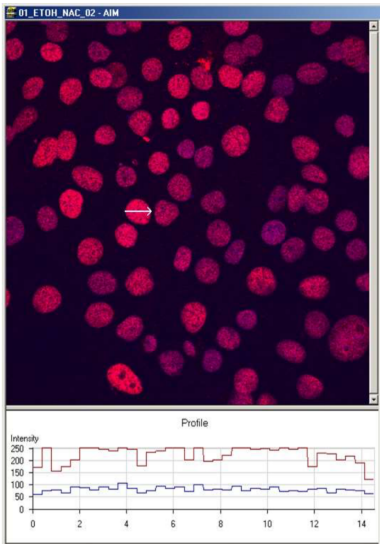

Figure 4.1: Exemplaric figure of cell nucleus analysis using immunofluorescence. Linescan and data record by laser scanning mikroscope (Zeiss LSM 510). Scan way (in μm) with 1000 measuring points each and detection of intensity of Alexa 555 respectively DRAQ5 with consecutive tabular account. Analysis of at least 50 cell cores of each treatment group.

We were able to identify certain epigenetic patterns. Histone modifications, in particular methylation and deacetylation, which are important for transcriptional

repression, were found persistently, but they exhibited substantial variations over time and depending on the applied dose of SM, so that a clear pattern could not be observed [16]. The results of the short-term and long-term *in vitro* investigations showed some similarities, like the formation of 5-mC and 5-hmC. In the long-term *in vitro* group the methylation status increased significantly over time and with subsequent cell passaging in low-exposure groups (0.5 μM and 1 μM) [16]. Also the skin samples exhibited changes in the DNA methylation status [123]. This puts DNA methylation under the main focus [127]. There are five different types of DNA methyltransferases (DNMTs), but only DNMT1, and DNMT3a and DNMT3b can transfer methyl groups from the usual donor S-adenosylmethionine to DNA [80, 152]. Interestingly the 'de novo' DNMTs DNMT3a and DNMT3b were not up-regulated under influence of SM, so that this task was probably assumed by DNMT1 [80, 133].

4.2 Epigenetical Therapeutical Targets

By identifying certain epigenetic patterns of early endothelial cells which were treated with SM *in vitro* and comparing them to the skin samples obtained, we demonstrated possible medical targets and similarities between different cell types [127]. There is no specific causal treatment available as of yet to approach the observed epigenetic changes. Histone modifications and DNA methylation present possible targets for eventual intervention. Histone-modifying enzymes, like e.g. histone deacetylases (HDACs), were found to play fundamental roles as regulators of differentiation and development of most cell types and interference in these processes naturally raises concern [96]. But nevertheless a transient manipulation is regarded to be useful [96]. Deacetylation of histones can therapeutically be blocked using histone deacetylase inhibitors (HDACis) like trichostatine A (TSA). TSA showed to be beneficial after intoxication with mechloretamine (MEC), a SM derivate; it may reactivate genes that were silenced by the agent and thereby preclude further damage [74]. Moreover, HDACis showed an effect on alternative splicing and might thereby regulate transcriptional elongation [28]. DNA methylation status is of special interest since our results presented significant changes under influence of SM. An imaginable intervention would be the application of DNA methyltranserase inhibitors (DNMTi). Described effects of this relatively new class of substances comprise the arrest of tumor growth and cell invasiveness as well as induction of differentiation [153]. By now the most common representatives are azacytidine (trade name: Vidaza®) and decitabine (DAC, trade name: Dacogen®). Azacitidine was approved 2004 by the FDA, decitabine followed in 2006 [91, 92]. They are used for treatment of myelodysplastic syndromes, chronic myelomonocytic leukemia and acute myeloid leukemia [30]. DAC (5-Aza-2-Deoxycitidin) - together with the local anaesthetic procaine - showed to prevent DNA methylation and deacetylation and was even able to counteract toxicity after SM intoxication *in vitro* [17]. Also natural sources of DNMTis including flavonoids like epigallocatechin-3-gallate (EGCG), curcumin or laccaic acid, are available and promising since unwanted side effects are less common [20, 153]. Silibinin, another phytochemical and natural supplier of DNMTis, was already used to mitigate SM toxicity - showing reduction of necrosis and proinflammatory effects - but epigenetic changes were not analyzed in parallel [10]. Hence, interventions with DNMTis could be a next step building upon our results. Confirmation that these substances are also relevant *in vivo* in order to treat SM intoxication has yet to be generated.

4.3 Limitations and Conclusion

Regarding the study design we used, it has to be stated that it holds some limitations. In the *in vitro* analysis we focused on endothelial progenitor cells as they are known to play an important role in the process of wound healing [142]. During our *in vitro* tests we received the information of the accidental exposure. Because of the level of difficulty of extracting a sufficient number of endothelial cells out of surgical skin samples we assessed the numerous and easy to identify keratinocytes in the *in vivo* sample. To prove clinical evidence though, a higher number of cases of *in vivo* samples would be needed and would ideally include the exact same cell type. Beside the probability that the encountered changes persist over at least several cell divisions, dynamic changes in epigenetic modifications over time were observed before, such that further longitudinal research designs are recommended [148]. Nevertheless, the partially congruent results strengthen the theory that modifications might appear universally even in different cell types after exposure [127]. Other alkylating agents such as Cbl showed similar effects in terms of strongly impairing endothelial cells and potentially leading to dysfunctional vascularization and abnormal wound healing, but intriguingly newer internal investigations of our working group showed distinct differences between the provoked epigenetic patterns of SM and Cbl [122, 134]. In conclusion it can be stated that we could corroborate the hypothesis that epigenetic mechanisms are involved in the pathophysiology of sulfur mustard. This is a first step for the future approach of epigenetic targets in the treatment of SM-caused damage. Stable molecular effects and specific targets could be identified to serve as a basis for further investigations. Ideally they should include *in vivo* studies with more gene-specific examinations of DNA and histone modulations. Further they should comprise therapeutic intervention groups to validate and expand knowledge of the discovered patterns and the clinical consequences thereof.

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