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Sulfur mustard resistant keratinocytes obtained elevated glutathione levels and other changes in the antioxidative defense mechanism

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Abstract

Background: Sulfur mustard (SM) is a potent blistering chemical warfare agent, which was first used in 1917. Despite the Chemical Weapons Convention, a use was recently reported in Syria in 2015. This emphasizes the importance to develop countermeasures against chemical warfare agents. Despite intensive research, there is still no antidote or prophylaxis available against SM.

Methods: The newly developed SM-resistant keratinocyte cell line HaCaT/SM was used to identify new target structures for drug development, particularly the adaptations in protective measures against oxidative stress. For this purpose, glutathione (GSH) and NAD(P)H levels, the effect of glutathione S-transferase (GST) inhibition as well as activation and expression of Nrf2, GST, glutamate cysteine ligase (GCL) and glutathione-disulfide reductase (GSR) as well as multi-drug resistance (MDR) proteins 1, 3 and 5 were investigated.

Results: The HaCaT/SM cells showed not only a better survival after treatment with SM or cytostatic drugs, but also hydrogen peroxide (H₂O₂). They exhibit more GSH even after SM treatment. Nrf2 levels were significantly lower. Inhibition of GST led to significantly decreased, activation to slightly higher IC₅₀ values after SM treatment and a lower expression of GST was observed. The cells also expressed less GCLC and GSR. Expression of MDR1, MDR3 and MDR5 was higher under control conditions, but less stimulated by SM treatment. An increased NADP⁺/NADPH ratio as well as higher NAD⁺ levels were shown.

Conclusion: In summary, an improved response of the resistant cell line to oxidative stress was observed. The underlying mechanisms are elevated GSH levels as well as lower expression of Nrf2

and its targets GCLC and GST as well as GSR and MDR1, MDR3 and MDR5. GST is an especially interesting target because its inhibition already induced a significant SM sensitivity. SM resistance also caused redox equivalent level differences. Taken together, these findings provide further insight into the mechanism of SM resistance and may open a window for novel therapeutic targets in SM therapy.

Keywords

Sulfur Mustard; Resistance; Keratinocytes; ROS; Oxidative Stress; Glutathione; Glutathione S-transferases; NAD(H); NADP(H)

1 Introduction

The chemical warfare agent sulfur mustard (SM) gained new public interest due to its use in terroristic attacks such as in Syria in August 2015 (OPCW Technical Secretariat, 2015). Despite its first use as a warfare agent 100 years ago, the mechanism of its effect on the organism is still not understood completely and no antidote or prophylaxis for SM exposure exists until now (Pohanka et al., 2017). SM shows acute toxicity in a variety of organs such as skin, eyes, respiratory system and the bone marrow (Thiermann et al., 2013). Proven pathologies are oxidative stress, apoptosis and inflammation. SM-induced oxidative stress results in depletion of low molecular mass antioxidants such as glutathione (GSH) and increase of reactive oxygen species (ROS) (Gould et al., 2009; Laskin et al., 2010; Poursaleh et al., 2012).

ROS scavengers such as N-acetyl cysteine (NAC) are already considered as potential new therapeutic approaches for SM treatment (Thiermann et al., 2013). GSH and NAC displayed protective effects against SM by reducing apoptosis, necrosis and interleukin levels (Balszuweit et al., 2016). NAC was also shown to prevent SM-induced activation of transient receptor potential ankyrin 1 (TRPA1) cation channel (Stenger et al., 2017). Antioxidant treatment directly after SM exposure seems to improve outcome (Steinritz et al., 2016).

Underlying mechanisms for a beneficial antioxidant treatment could be an altered expression of oxidative stress response genes. Oxidative stress is known to induce specific signal transduction pathways (Ray et al., 2012). One important stress-induced pathway involves the transcription factor nuclear factor (erythroid-derived 2)-like 2 (Nrf2), which regulates the transcription of antioxidant genes. Under physiological conditions, Nrf2 is localized in the cytoplasm and is degraded by ubiquitination. Oxidative stress prevents its degradation and Nrf2 is translocated into the nucleus, where it binds to antioxidant-response-elements (AREs) after dimerization with small Maf proteins. Nrf2 targeted ARE-regulated genes include glutamate-cysteine ligase (GCLC), GST family and multidrug resistance-associated proteins (MRPs) among others (Maher et al., 2007; Ray et al., 2012; Solis et al., 2002). Nrf2 was already shown to be involved in radio-resistance of cancer cell lines (Jayakumar et al., 2014; Singh et al., 2010) and it might be involved in CEES and nitrogen mustard toxicity (Abel et al., 2011; Udasin et al., 2016).

The synthesis, regeneration and conjugation of GSH are examples of Nrf2-influenced pathways. The expression of antioxidants and ROS-producing genes was significantly

altered in lung tissue of SM-exposed patients (Tahmasbpour et al., 2016). Overexpression of the glutathione S-transferase (GST) variants GSTA1, GSTM1 and GSTP1 was shown in the human airway wall as a long-term response to SM (Nourani et al., 2014).

Another important protein family regulated by Nrf2 is MRP, which belongs to the ATP-binding cassette transporters (ABC transporters) superfamily. They use the energy of ATP hydrolysis for active transport of various substrates, drugs and other xenobiotics either conjugated or unconjugated across the cell membrane. The spectra of substrates are similar for the most extensively-studied *ABCB1* protein (P-glycoprotein, MDR1) and multidrug resistance protein 1 and 2 (MRP1/2, encoded by *ABCC1/2*) and they transport a wide variety of anticancer drugs such as methotrexate, cisplatin, vincristine, doxorubicin, VP-16 and topotecan (Leslie et al., 2005). Reversing the ABC-transporter-mediated multi-drug resistance is a promising strategy in cancer therapy. SM might also be transported via specific ABC transporters, due to its similarity to nitrogen mustards used in chemotherapy as it was recently demonstrated that sensitivity to nitrogen mustard was regulated via Nrf2-mediated MRP1 (Udasin et al., 2016).

SM is able to alkylate DNA bases leading to intra- and inter-strand cross-links which results in DNA strand breaks. In consequence, poly(ADP-ribose) polymerase (PARP) is activated which depletes NAD^+ while using it as a cosubstrate for ADP-ribosylation. This results in glycolysis inhibition and NADP^+ -dependent pentose phosphate pathway stimulation. The accumulation of glucose-6-phosphate leads to induction and secretion of proteases resulting in nuclear and cytoplasmic damage (Graham et al., 2005). The NAD^+ depletion often mentioned in the literature could not be validated by other studies (Mangerich et al., 2016). Otherwise, niacin, the vitamin from which NAD^+ and NADP^+ are synthesized, showed protective effects against DNA-damaging agents (Jacobson et al., 2007). Taken together, cellular redox equivalents seem to play important roles in SM toxicity.

Previously, the human keratinocytes cell line HaCaT/SM was described as SM-resistant cell line (Schmidt et al., 2016). First results showed that this cell line underwent significantly reduced DNA alkylation and increased clonogenicity potential upon SM exposure as well as showed a different miRNA expression pattern and is also resistant to a variety of cytostatic drugs currently used in chemotherapy (Rothmiller et al., 2017; Schmidt et al., 2016; Schmidt et al., 2017; Wolf et al., 2016). The understanding of the underlying mechanism of SM resistance may open a window for new therapeutic approaches. Herein, oxidative stress as one of the main mechanisms of action is of central importance and thus it was investigated whether the SM-resistant HaCaT/SM cell line exhibited an improved antioxidant stress response and what biochemical mechanisms laid behind.

2 Materials and Methods

2.1 Cell culture and sulfur mustard exposure

HaCaT cells as well as the SM-resistant keratinocytes cell line HaCaT/SM (Schmidt et al., 2016) were cultivated in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 5 % FBS at 37 °C in a humidified atmosphere containing 5 % CO_2 using standard cell culture flasks (75 and 175 cm^2). For passaging, the cells were detached with 0.05 % Trypsin

and 1 mM EDTA for 5 min and resuspended in fresh medium at least twice a week. The SM-resistant cell line HaCaT/SM was generated from HaCaT cells as described before (Schmidt et al., 2016) and exposed to 7.2 μ M SM once a week. Cell number was determined by CASY® Cell Counter.

SM (bis-[2-chloroethyl]sulfide; purity > 99 %, confirmed by NMR) was made available by the German Ministry of Defense. Prior the experiments, pure SM (8 M) was prediluted in ethanol. Final dilution was added to medium with 0.5 % maximum EtOH volume and vehicle controls were included. Exposure and incubation of cells were performed under a fume hood to meet safety regulations.

2.2 Cell survival assay

For resistance determination, 40,000 cells were seeded per well in a clear bottom-flat 24-well plate (Greiner) including medium control as blank and were grown overnight. The cells were exposed to increasing concentrations from 0.2 to 500 μ M SM including vehicle control for 5 days. Glutathione S-transferase (GST) inhibitors ezatiostat (TLK199, Cayman Chemical) and ethacrynic acid (Sigma) were added to medium in final concentrations of 400 nM and 600 nM, respectively. Stock solutions of both inhibitors were prepared in DMSO with 10 mg/mL. After pre-dilution in 100 % EtOH, GST activator ethoxyquin (Sigma) was used with 24 μ M final concentration as a pre-treatment for 24 hours. For testing resistance against H₂O₂, concentrations between 0.24 and 80,000 μ M were used including medium control for 5 days. Therefore, 30 % H₂O₂ (Sigma) was pre-diluted in sterile ddH₂O. After exposition over 5 days, cells were washed with medium and XTT staining solution (Sigma) was prepared by mixing 5 mL XTT labeling reagent with 0.1 mL electron-coupling reagent per plate. 400 μ L medium with 200 μ L of XTT staining solution were added per well and incubated for 6 h. Extinction was measured at 450 nm with reference at 630 nm. All conditions were tested in biological triplicates.

2.3 GSH/GSSG Assay

10,000 cells were seeded per well in a white 96-well plate with a clear bottom (Greiner) including medium control and grown overnight. Cells were exposed to 1.8, 3.6 or 7.2 μ M SM including ethanol control for 24 h in 100 μ L final volume. Assay was performed according to user's manual using GSH/GSSG-Glo Assay (Promega). Luminescence was recorded with 1000 ms integration interval using the plate reader infinite M200 Pro (Tecan). All conditions were tested in biological triplicates.

2.4 Intracellular real-time GSH determination with RT-probe

Lyophilized RealThiol (RT) probe was kindly provided by Jin Wang and Xiqian Jiang (Jiang et al., 2017). 5 mM stock solution was prepared by dissolving lyophilized RT-probe in DMSO and stored aliquoted at -80 °C. Immediately before starting the assay, working concentration of 1 μ M was adjusted by dilution with DMSO (50 times) and fresh medium (100 times). 1 mL working concentration of RT-probe was sufficient for 2×10^5 cells.

HaCaT and HaCaT/SM cells were diluted to yield 1×10^5 cells per mL and one mL each was transferred to a new reaction tube. RT-probe was diluted to working solutions directly to

the cell suspension. The suspensions were mixed and stained for 10 to 15 min at room temperature. Afterwards, 50 μ L of stained cell suspension was transferred into a black 96-well plate with a clear bottom (Greiner). Baseline was recorded every 2 min for 12 cycles using a plate reader infinite M200 Pro at 37 °C and 5 % CO₂ (Tecan). Cells were exposed to 100 μ M SM and recording was continued until 400 min. Parameters determined were F₄₀₅ (405 nm excitation, 430/470 nm emission) and F₄₈₈ channel (488 nm excitation, 510–660 nm emission) and GSH concentration was determined as F₄₀₅/F₄₈₈ ratio.

2.5 In Cell Western

In Cell Western assay is a new approach to visualize proteins within cells or tissues (Egorina et al., 2006). For In Cell Western, 20,000 cells were seeded per well in a black 96-well plate with clear bottom (Greiner) and grown until almost confluent. Cells were exposed to 1.8 μ M, 3.6 μ M, 7.2 μ M or 15 μ M SM as well as ethanol as control for 3 days. For the following, washing with PBS, 0.1 % Tween-20 in PBS (PBS-T) and H₂O was always performed with 100 μ L volume, all other steps with 50 μ L. Cells were washed once with PBS and fixed with 4 % PFA in PBS at 4 °C for 30 min. Wells were washed three times with PBS and cells were permeabilized with 0.1 % Triton X-100 (Sigma) in PBS at 4 °C for 5 min. After washing three times with PBS, proteins were blocked with Odyssey Blocking Buffer (LI-COR) at RT for 30 min. Primary antibodies were diluted in Odyssey Blocking Buffer and incubated at 4 °C overnight. The following primary antibodies were used: Anti-P-Glycoprotein (MDR1) 1:1000 (Sigma), Anti-MDR3 P-Glycoprotein (MDR3) 1:20 (Sigma), Anti-ABCB5 (MDR5) 1:1000 (antibodies online), glutamate-cysteine ligase (GCLC) 1:1000 (antibodies online), glutathione reductase antibody (GSR) 1:1000 (antibodies online), glutathione S-transferase pi 1 (GSTP1) 1:1000 (antibodies online) and anti-Nrf2 1:1000 (Abcam). After washing three times with PBS-T, secondary antibodies Goat 800 CW, Mouse 800 CW or Rabbit 800 CW 1:1000 (LI-COR) and DRAQ5 1:1000 (Biostatus) were added together in Odyssey Blocking Buffer at RT for 90 min. After washing three times with PBS-T, blot development was performed in PBS with Odyssey IR scanner (LI-COR). Background (wells with cells and same treatment but without primary antibody) was subtracted, expression levels were normalized to cell number via DRAQ5 and HaCaT controls set to 100 %. Each In Cell Western was performed in biological triplicates.

2.6 NAD⁺/NADH and NADP⁺/NADPH Assay

For each cell line, four T75 flasks were grown until almost confluent and afterwards exposed to 1.8 μ M, 3.6 μ M or 7.2 μ M SM and ethanol as control, for 24 h. Cells were detached with trypsin and total cell numbers were counted. Cells were pelleted at 4 °C, washed once with cold PBS and were then resuspended in cold PBS. Per condition 5×10^5 cells were transferred to a new tube. Cells were pelleted, shock-frozen without supernatant in liquid nitrogen and stored at –80 °C until assay performance.

NAD/NADH determination was performed according to user's manual using NAD/NADH Quantitation Kit (Sigma). Briefly, cell pellets were thawed on ice and 1 mL NADH/NAD extraction buffer was added per tube. Extraction was performed using a homogenizer (3×5 s, 25 % intensity). NADH/NAD extraction buffer was added to each tube to reach 5×10^5 cells per mL. Tubes were vortexed for 10 sec and centrifuged at 13,000 g for 10 min at 4 °C.

Cell lysates were deproteinized by Amicon Ultra 0.5 mL Ultracel 10K cut-off filter (Merck Millipore) by centrifugation at 14,000 g for 15 min at 4 °C. Deproteinized lysate was incubated at 60 °C for 30 min, shortly cooled on ice and centrifuged at 14,000 g for 2 min at 4 °C. 50 µL of supernatant were used and 25 µL of not heated deproteinized sample for NAD total determination. NADH standard was dissolved in 200 µL DMSO to yield 1 mM concentration. Between 2 and 10 µL of a 1:100 dilution in NAD/NADH extraction buffer were added to each well. All samples and standards were added up to 50 µL with NAD/NADH extraction buffer. 100 µL of NAD Cycling Enzyme Mix in NAD Cycling Buffer were added per well and incubated for 5 min at RT shaking. 10 µL NAD Developer were added per well and the absorbance at 450 nm (A_{450}) was recorded until 100 pmol standard reached an A_{450} of 2.0. Experiments were run in triplicates.

NADP/NADPH quantitation was performed according to user's manual using NADP/NADPH Quantification Kit (Sigma) and similar to NAD/NADH kit. Cell pellets were lysed in NADPH/NADP extraction buffer and sample preparation was performed as described above. NADPH standard was dissolved in 200 µL DMSO, further diluted 1:100 in NADPH/NADP extraction buffer and between 2 and 10 µL were added per well. 50 µL were added for NADPH and 25 µL for NADP total determination. All wells were added up to 50 µL with NADPH/NADP extraction buffer. 100 µL of NADP cycling enzyme mix in NADP cycling enzyme buffer were added per well and incubated for 5 min at RT shaking. 10 µL NADP developer were added per well and A_{450} was recorded until maximal absorbance of 2.0. Experiments were run in triplicates.

2.7 Statistic methods

All data were evaluated with GraphPad Prism version 5. IC_{50} values were determined by nonlinear regression and compared by extra sum-of-squares F test. Expression and substrate levels were compared by two-way ANOVA with Bonferroni post tests for comparison with controls. p values less than 0.05 were considered significant.

3 Results

3.1 Improved response against oxidative stress and differences in intracellular glutathione levels

3.1.1 Resistance against hydrogen peroxide—The established SM-resistant keratinocytes cell line HaCaT/SM (Schmidt et al., 2016) is not only resistant against SM, but also against a variety of alkylating cytostatic drugs currently used in chemotherapy (Schmidt et al., 2017). The continuous treatment with SM resulted in an increased intracellular level of reactive oxygen species (ROS) and therefore, it was hypothesized that HaCaT/SM might also be resistant against ROS. To test this hypothesis, the cells were exposed to increasing doses of hydrogen peroxide (H_2O_2).

Treatment with H_2O_2 showed that HaCaT/SM were able to tolerate higher doses than HaCaT cells (Figure 1). The resistant cell line displayed a significantly higher IC_{50} value of about 1.2 fold with 685.2 ± 1.0 µM compared to HaCaT with 556.5 ± 1.1 µM.

3.1.2 Differences in glutathione levels—Levels of glutathione were determined 24 h after SM treatment. For exposition, 1.8 μ M, 3.6 μ M or 7.2 μ M SM were used and ethanol as control. Total glutathione and oxidized glutathione (GSSG) of cell lysates were directly determined by the assay and therefore reduced (GSH) and the GSH/GSSG ratio could be calculated.

HaCaT/SM cells contained significantly more ($9.7 \pm 0.1 \mu$ M) total glutathione than HaCaT cells ($5.8 \pm 0.9 \mu$ M). With increasing SM concentrations, the total glutathione concentration decreased in both cell lines. At 7.2 μ M SM, HaCaT ($4.0 \pm 0.2 \mu$ M) and HaCaT/SM ($8.7 \pm 0.1 \mu$ M) showed a significant decrease compared to controls, respectively. The same trend was observed for GSH concentrations. GSSG concentrations were also higher in HaCaT/SM than in HaCaT cells and they decreased with increasing SM concentrations in both cell lines. The GSH/GSSG ratio was throughout significantly higher in HaCaT.

3.1.3 Live recording of glutathione levels using RealThiol (RT) probe—The RT-probe was recently developed and reported to be the first fluorescent probe to quantitatively monitor the glutathione concentration in living cells in real-time (Jiang et al., 2017). Therefore, the fluorescent intensities at the excitation wavelengths 405 and 488 nm were determined for RT-probe and the GSH-RT-probe adduct, respectively. The ratio of F_{405}/F_{488} was then used as indicator for GSH concentration (Figure 3).

After staining, the basal F_{405}/F_{488} ratio was determined. The mean of the F_{405}/F_{488} ratio in HaCaT/SM was 0.87 ± 0.45 compared to 0.83 ± 0.32 in HaCaT, showing no significant difference between basal GSH levels in cell suspensions. Addition of 100 μ M SM resulted in a rapid decrease in GSH levels in both cell lines within about 5 min. Immediately afterwards the GSH levels increased up to a maximum at 100 and 120 min for HaCaT and HaCaT/SM, respectively. In HaCaT cells, the GSH concentration was reduced, then stable from 150 to 250 min and raised again afterwards. In contrast, in HaCaT/SM cells there was only a very small decrease from 120 to 200 min and afterwards the GSH concentration raised linear and above the basal levels.

3.2 Underlying cellular mechanisms

3.2.1 Reduced Nrf2 expression levels—The elevated GSH levels (4.1.2) in the resistant cell line suggest a better stress response. Therefore, the underlying cellular mechanisms of these elevated GSH levels were investigated. An important central regulator for oxidative stress is Nrf2. It is a basic leucine zipper protein and a transcription factor that regulates the expression of antioxidant proteins to protect against oxidative damage.

HaCaT and HaCaT/SM showed comparable levels of Nrf2 under control conditions ($118 \pm 54 \%$ vs. $100 \pm 42 \%$; Figure 4). In contrast, exposure of the cells to 7.2 or 15 μ M SM resulted in an increased Nrf2 expression. In HaCaT/SM ($223 \pm 53 \%$ at 7.2 μ M SM), Nrf2 expression levels were significant lower compared to HaCaT cells ($443 \pm 22 \%$ at 7.2 μ M SM). The higher dose of 15 μ M SM resulted in a slight increase of Nrf2 expression in HaCaT/SM with $287 \pm 36 \%$ and in HaCaT with $499 \pm 15 \%$. Overall, the Nrf2 expression under the influence of SM was higher than under control conditions and significantly higher in the control cell line HaCaT under influence of SM.

3.2.2 Reduced expression of enzymes involved in GSH cycle—The elevated GSH levels (4.1.2) in the resistant cell line might be a consequence of altered protein expression of enzymes involved in the GSH cycle. Therefore, the expression levels of the glutathione S-transferase P1 (GSTP1), glutathione reductase (GSR) and glutamate-cysteine ligase (GCLC) were investigated (Figure 5).

The resistant HaCaT/SM cells showed significantly lower expression of GCLC under control conditions as well as after SM treatment. In absence of SM, the resistant cells expressed about 30 % less GCLC than HaCaT (71 ± 10 % vs. 100 ± 8 %). SM treatment slightly induced GCLC expression concentration-dependent in both cell lines, with a more pronounced effect in HaCaT.

GSR levels were also about 25 % lower in HaCaT/SM in absence of SM (74 ± 25 % vs. 100 ± 17 %). Treatment with the applied SM doses did not increase the expression, in fact it may even been decreased. The significance of the lower expression was shown for all conditions except at $7.2 \mu\text{M}$ SM.

Under control conditions, there was no difference in GSTP1 expression between both cell lines. Upon SM treatment, a concentration-dependent increase in GSTP1 expression could be observed in HaCaT and HaCaT/SM cells, but this was more pronounced in HaCaT cells. The expression levels were significantly higher in HaCaT cells at all SM concentrations tested.

3.2.3 Influence of GST inhibition and activation on SM resistance—Glutathione S-transferases (GSTs) conjugate xenobiotics with GSH for detoxification. The aim was to determine if inhibition or activation of this important anti-oxidative protein family influences SM resistance. Therefore, cell survival was tested using the XTT assay after SM treatment with concentrations between 0.2 and $500 \mu\text{M}$ SM for 5 days. Cell survival was determined in presence and absence of two different glutathione S-transferase inhibitors, ethacrynic acid (inhibition of different GSTs) and ezatiostat (GSTP1–1 selective). Moreover, the activation of GSTs by ethoxyquin as 24 h pretreatment was also investigated for its influence on SM resistance.

Cell survival curves for HaCaT (Figure 6 A) and HaCaT/SM (Figure 6 B) are shown in presence and absence of the inhibitors ezatiostat and ethacrynic acid. While HaCaT cells showed an IC_{50} of $5.9 \pm 1.1 \mu\text{M}$, treatment with ethacrynic acid reduced the IC_{50} level to $2.5 \pm 1.1 \mu\text{M}$ and treatment with ezatiostat reduced the IC_{50} level to $2.6 \pm 1.1 \mu\text{M}$. Apart from that, HaCaT/SM (Figure 6 B) showed an IC_{50} of $58.4 \pm 1.1 \mu\text{M}$. Treatment with ethacrynic acid reduced the IC_{50} level to $40.1 \pm 1.1 \mu\text{M}$ and treatment with ezatiostat reduced the IC_{50} level to $36.1 \pm 1.1 \mu\text{M}$. Both inhibitors induced a significant SM sensitivity in both cell lines investigated.

Treatment of HaCaT (Figure 6 C) and HaCaT/SM (Figure 6 D) with GST activator ethoxyquin increased slightly but not significantly the cell survival after SM treatment. The IC_{50} increased from $6.5 \pm 1.2 \mu\text{M}$ to $7.8 \pm 1.3 \mu\text{M}$ in HaCaT and from $34.5 \pm 1.1 \mu\text{M}$ to $51.5 \pm 1.1 \mu\text{M}$ in HaCaT/SM.

3.2.4 Reduced expression of MDR transporters—The following three different members of multidrug resistance (MDR) transporters were investigated: P-glycoprotein (MDR1, encoded by *ABCB1*), MDR3 (encoded by *ABCB4*) and MDR5 (encoded by *ABCB5*).

The levels of MDR1 (Figure 7 A) increased in both cell lines under increasing SM concentrations. HaCaT showed a lower expression under control conditions ($100 \pm 3 \%$) than HaCaT/SM ($142 \pm 70 \%$). The increase of MDR1 expression was more promoted in HaCaT than in HaCaT/SM cells but expression differences between both cell lines were not significant.

MDR3 level (Figure 7 B) was significantly higher in HaCaT/SM ($136 \pm 29 \%$) than in HaCaT ($100 \pm 8 \%$) cells under control conditions. For both cell lines an increase in MDR3 expression was shown to be correlated with increasing SM concentrations. This increase was more promoted in HaCaT (1.8 μ M: $157 \pm 5 \%$; 3.6 μ M: $229 \pm 4 \%$; 7.2 μ M: $261 \pm 5 \%$) than in HaCaT/SM (1.8 μ M: $153 \pm 16 \%$; 3.6 μ M: $184 \pm 5 \%$; 7.2 μ M: $215 \pm 4 \%$). Therefore, HaCaT cells showed higher MDR3 expression under SM exposure, which was significantly higher at 3.6 μ M and 7.2 μ M SM.

MDR5 (Figure 7 C) level in HaCaT/SM ($156 \pm 54 \%$) was significantly higher than in HaCaT cells ($100 \pm 5 \%$) under control conditions. HaCaT showed an SM-concentration dependent increase in MDR5 level. Under SM exposure, HaCaT cells even showed a significant higher MDR5 level at 3.6 μ M and 7.2 μ M SM.

3.3 Changes in levels of redox equivalents

3.3.1 NAD⁺ and NADH—Intracellular parameters for NAD⁺ and NADH were assessed 24 h after SM treatment. Total NAD and reduced NADH were directly determined in cell lysates by the assay. With these values oxidized NAD⁺ and NAD⁺/NADH ratios could be calculated (Figure 8).

HaCaT/SM showed significantly higher amounts of total NAD than HaCaT cells. These relations were also found for intracellular NADH. HaCaT/SM contained also significantly more NAD⁺ (0 μ M: 75.1 ± 0.8 pmol; 1.8 μ M: 55.2 ± 0.2 pmol; 3.6 μ M: 102.1 ± 1.4 pmol; 7.2 μ M: 76.0 ± 3.4 pmol). HaCaT cells contained 29.4 ± 0.1 pmol NAD⁺ at control conditions and slightly increased values when treated with SM (1.8 μ M: 36.6 ± 0.5 pmol; 3.6 μ M: 40.0 ± 0.5 pmol; 7.2 μ M: 31.2 ± 0.2 pmol). The NAD⁺/NADH ratio in HaCaT cells was only significantly higher under control conditions (215.1 ± 145.9 vs. 21.5 ± 1.1), while no significant difference could be detected after SM exposure.

3.3.2 NADP⁺ and NADPH—Intracellular parameters for NADP⁺ and NADPH were determined 24 h after SM treatment. Total NADP and reduced NADPH were directly determined after cell lysis by the assay. With these values oxidized NADP⁺ and NADP⁺/NADPH ratios could be calculated (Figure 9).

HaCaT/SM contained significantly more NADP⁺ than HaCaT cells. There was one exception, i.e. at 3.6 μ M SM. The NADP⁺ amount was higher under SM treated than under

control conditions in HaCaT cells and the levels first raised, then dropped with increasing SM concentrations (0 μ M: 2.7 ± 0.1 pmol; 1.8 μ M: 4.0 ± 0.1 pmol; 3.6 μ M: 3.6 ± 0.0 pmol; 7.2 μ M: 3.0 ± 0.1 pmol). This dose-dependent response was parallel but invers in HaCaT/SM cells (0 μ M: 9.0 ± 0.1 pmol; 1.8 μ M: 7.8 ± 0.1 pmol; 3.6 μ M: 3.3 ± 0.1 pmol; 7.2 μ M: 8.9 ± 0.1 pmol). The NADPH amount was significantly higher in HaCaT than in HaCaT/SM cells. Therefore, the NADP⁺/NADPH ratio was significantly higher in HaCaT/SM than in HaCaT cells under control and SM treated conditions.

4 Discussion

The SM-resistant keratinocytes cell line HaCaT/SM (Schmidt et al., 2016) was shown to be also resistant against various cytostatic drugs used in chemotherapy (Schmidt et al., 2017). Herein, it could be demonstrated that these cells are likewise able to tolerate higher doses of hydrogen peroxide (H₂O₂) and contained elevated intracellular glutathione (GSH) levels, which suggested an enhanced ability to cope with oxidative stress compared to HaCaT cells. The underlying molecular mechanisms might be derived from lower levels of the transcription factor Nrf2. It could be shown that the Nrf2 target proteins GCLC and GST as well as GSR and MDR1, MDR3 and MDR5 were differentially expressed. Moreover, altered levels of reduction equivalents were determined.

4.1 Sulphur mustard induced oxidative stress, improved response and differences in intracellular glutathione levels

In the literature SM toxicity is correlated with oxidative stress. A maximum of oxidative stress production was found 2–6 h after CEES exposure, mainly by production of O₂⁻ (Inturi et al., 2011). In human bronchial epithelial cells, a peak in oxidative stress was found 12 h after CEES exposure (Gould et al., 2009). Topical CEES exposure in SKH-1 hairless mice showed an increase in oxidative stress between 3 and 168 h after exposure to 1–2 mg CEES (Pal et al., 2009). In conclusion, all studies propose a time- and concentration-dependent oxidative stress profile after CEES or SM exposure.

The resistance against SM in HaCaT/SM might be at least in part developed by an improved handling of oxidative stress. This hypothesis was tested using cell survival curves after H₂O₂ treatment. Indeed HaCaT/SM tolerated a significant higher level of H₂O₂ compared to HaCaT controls.

Glutathione (GSH) is considered as the most important cellular defense mechanism against oxidative stress (Diaz-Vivancos et al., 2015). In HaCaT/SM cells was significantly more total glutathione present, throughout. The total glutathione levels decreased with increasing SM concentrations, which was significant in both cell lines. The RealThiol (RT) probe, which allowed the quantitative real-time determination of glutathione concentrations in living cells (Jiang et al., 2017), showed a better response of the HaCaT/SM cells to SM treatment which resulted in a higher level of GSH than HaCaT cells at about 120 min and thereafter. The fast decrease of GSH within minutes after treatment with 100 μ M SM shows also a very fast reaction to SM, comparable to findings that proved fast activation of TRPA1 after SM treatment, a mechanism different from the alkylating mechanism (Stenger et al., 2017).

The decrease of intracellular GSH after SM or CEES treatment was already described before. After 24 h treatment of HaCaT with 0.5 mM CEES, GSH levels decreased to 26 % compared to control (Tewari-Singh et al., 2011). In our experiments, the intracellular GSH concentrations in HaCaT cells decreased to about 71 % at 7.2 μ M SM after 24 h of treatment. Hence, it appears that the decrease in GSH levels is concentration dependent and may lead to complete depletion if the concentration of alkylating agent is increased adequately.

Summarizing, SM showed an effect on intracellular GSH and the resistant cell line HaCaT/SM contained more GSH and therefore more possible scavenging molecules indicating that GSH may play a role in the resistance against SM.

4.2 Underlying cellular mechanisms

4.2.1 Reduced Nrf2 expression levels—Nrf2 is an important central regulator of oxidative stress that controls the expression of antioxidant proteins to protect against oxidative damage (Ray et al., 2012). While Nrf2 levels were comparable in absence of SM, treatment with 7.2 μ M and 15 μ M SM resulted in a concentration dependent increase in Nrf2 expression which was more pronounced in HaCaT. Therefore, the significantly lower Nrf2 expression in the resistant cells may indicate a lower need for antioxidative proteins.

Nrf2 seems to play important roles in SM toxicity, because the Nrf2 inducer CDDO-Me increased the survival of human keratinocytes (NCTC2544) 3-fold after CEES exposure (Abel et al., 2011). Moreover, the overexpression of Nrf2 in chemo- and radioresistance could be shown (Jayakumar et al., 2014; Singh et al., 2010).

4.2.2 Reduced expression of enzymes involved in GSH cycle—The elevated GSH levels in the resistant cell line might be a consequence of altered protein expression of enzymes involved in the GSH cycle, which are also regulated by Nrf2 (Solis et al., 2002). Expression of glutathione S-transferase P1 (GSTP1) catalyzes the conjugation of xenobiotics with GSH. The glutathione reductase (GSR) reduces the oxidized GSSG to two molecules of reduced GSH, and glutamylcysteine ligase (GCLC) is the rate-limiting enzymes in the GSH synthesis (Franklin et al., 2009). All three enzymes were found to be significantly lower expressed in the resistant HaCaT/SM cell line three days after SM exposure and also under control conditions (except GSTP1 under control conditions). The overall reduced expression in HaCaT/SM is in line with the lower levels of Nrf2. A possible explanation therefore is that the resistant cells exhibit less oxidative stress, resulting in lower Nrf2 activation and in the following to a lower GSTP1, GSR and GCLC activation.

4.2.3 Influence of GST inhibition and activation on SM resistance—Moreover, the influence of inhibition and activation of GSTs on the cell survival after SM treatment was determined. Therefore, the inhibitors ezatiostat (TLK199, potent and selective for GSTP1-1) and ethacrynic acid (less selective, best studied GST inhibitor) as well as the activator ethoxyquin were used (Henson et al., 2001; Laborde, 2010). Both GST inhibitors ezatiostat and ethacrynic acid induced a significant SM sensitivity not only in HaCaT, but also in HaCaT/SM cells. The reduction of IC₅₀ values was about 2.2 and 2.4-fold in HaCaT as well as 1.6 and 1.5-fold in HaCaT/SM for ezatiostat and ethacrynic acid, respectively. In

contrast, GST activation by ethoxyquin treatment resulted in a slightly but not significantly increased SM resistance of 1.2-fold in HaCaT and 1.5-fold in HaCaT/SM. Additionally, ethoxyquin treatment influenced the slope of the curves which resulted in an improved cell survival at SM doses between about 10 and 100 μ M in both cell lines.

4.2.4 Reduced expression of MDR transporters—The expression of MDR1, MDR3 and MDR5 was elevated under control conditions in the resistant cell line and SM exposure induced the expression in both cell lines. The increase in expression correlated in both cell lines with the SM concentration and may be associated with the attempt of the cells to actively transport SM or SM-conjugated substances out of the cell. This is in line with the result, that multidrug resistance-related proteins (MRP1 and MRP2) are known to transport metabolites of alkylating anticancer agents including chlorambucil and cyclophosphamide (Qiu et al., 2004; Smitherman et al., 2004). Interestingly, the SM-induced MDR expression is more pronounced in HaCaT cells. It is possible that the constitutively higher levels at control conditions in HaCaT/SM might be almost enough to reduce the pathophysiological effects and a less expression stimulation might be sufficient. The reduced expression might also be influenced by the lower Nrf2 levels accordingly to the findings that Nrf2 is able to stimulate the coordinated activation of MRP2, MRP3 and MRP4 (Maher et al., 2007).

4.3 Changes in levels of redox equivalents

4.3.1 NAD⁺ and NADH—Theories of SM-induced vesication includes depletion of intracellular NAD⁺ due to activation of PARP leading to inhibition of cellular energy metabolism, protease release and cell death (Kehe et al., 2008; Martens and Smith, 2008). Human epidermal keratinocytes (HEK) cells showed a significant decrease of intracellular NAD⁺ at 100 and 500 μ M SM after 24 h exposure to 20 % and 55 % compared to control, respectively (Martens and Smith, 2008). Skin lesion on hairless guinea pigs after SM exposure showed a decrease of skin NAD⁺ content to a minimum (20 % of control) at 16 h (Yourick et al., 1993).

In contrast to the literature data, a decrease or even depletion in NAD⁺ levels was not observed under SM exposure in our study. Moreover, the resistant cells showed significantly higher levels of NAD⁺, NADH and therefore total NAD than the sensitive cells. Supporting our data, the NAD⁺ depletion often described in the literature could also not be validated by even treatment with up to 3 mM CEES and 24 h (Mangerich et al., 2016). The NAD⁺ decrease seems to be concentration dependent and might be achieved by increasing the SM doses adequately. The higher NAD⁺ levels in the resistant cells are in line with the observation that boosted NAD⁺ levels reduced SM-induced pathology in animal experiments. Pretreatment with niacinamide reduced the microvesication formation after SM treatment by 50 % in hairless guinea pigs (Yourick et al., 1991). Another study shows that niacin has protective effects against DNA-damaging agents (Jacobson et al., 2007).

4.3.2 NADP⁺ and NADPH—The resistant cell line HaCaT/SM contained more NADP⁺ and less NADPH than HaCaT. Therefore the NADP⁺/NADPH ratio is higher in HaCaT/SM cells 24 h after SM exposure as well as under control conditions.

In the literature, the influence of SM on NADP(H) levels are poorly characterized. Skin lesion on hairless guinea pigs after SM exposure were correlated with an increase in skin NADP⁺ content (260 % of control) between 1 and 2 h and returned to control levels at 4 h. It was proposed that this indicates an early phase of cellular oxidative stress that may contribute to SM-induced dermal pathogenesis (Yourick et al., 1993). This increase in NADP⁺ amount after SM exposure was also slightly observed in this study. This increase may not only be time- but also concentration-dependent.

NADPH is used in anabolic reactions such as lipid and nucleic acid synthesis. It is also necessary to reduce GSSG to GSH catalyzed by GSR (Stanton, 2012). The significantly lower levels in HaCaT/SM might indicate that less NADPH is needed to reduce GSSG due to the improved stress response induced by SM resistance.

Concluding, the results showed that oxidative stress is reduced in the SM-resistant HaCaT/SM cell line due to an improved stress response. The biochemical background are elevated GSH levels as well as lower expression of Nrf2 and its targets GCLC and GST as well as GSR and MDR1, MDR3 and MDR5. GST is an especially interesting target because its inhibition already induced a significant SM sensitivity. SM resistance also led to redox equivalent level differences. Taken together, these findings provide further insight into the mechanism of SM resistance and may open a window for new therapeutic targets in SM therapy.

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Figure 1: Cell survival of HaCaT/SM and HaCaT after H₂O₂ treatment.

Cell survival of HaCaT (black) and HaCaT/SM cells (dashed) after hydrogen peroxide (H₂O₂) exposure with 0.24 to 80,000 μM H₂O₂ was tested for 5 days via XTT assay (Roche). IC₅₀ values are 556.5 ± 1.1 μM (HaCaT) and 685.2 ± 1.0 μM (HaCaT/SM). The difference was significant ($p = 0.0070$).

Figure 2: Influence of SM and SM resistance on glutathione levels.

Differences in glutathione (GSH), glutathione disulfide (GSSG), GSH/GSSG ratio and total glutathione levels between cell lysates of HaCaT (white) and HaCaT/SM (black) 24 hours after SM exposure are shown. Both cell lines were either exposed to EtOH controls (0 μM) or 1.8, 3.6 and 7.2 μM SM. Parameters were determined by the GSH/GSSG-Glo Assay (Promega) according to user's manual. The results are shown as mean $\pm$ SD of triplicates. Significant differences ($p < 0.05$) are indicated by the capped line.

Figure 3: Quantitative real-time recording of GSH after SM exposure.

Free GSH reacts with RealThiol (RT) probe (F₄₀₅) to a GSH-RT-probe adduct (F₄₈₈) and the F₄₀₅ to F₄₈₈ ratio was used to quantify the GSH level (Jiang et al., 2017). Cell suspensions of HaCaT/SM (grey) and HaCaT (black) were recorded 20 min to determine basal GSH concentration. The recording was continued after addition of 100 μM SM, while cells were kept at 37 °C and 5 % CO₂. GSH levels in HaCaT/SM raised faster and above the control level after 400 min, whereas they only reached the control levels in HaCaT.

Figure 4: SM exposure induced Nrf2 expression and the effect was stronger in SM sensitive cells. Nrf2 levels shown as mean $\pm$ SD were determined by In Cell Western for HaCaT (white) and HaCaT/SM (black). Both cell lines were either exposed to EtOH controls (0 μ M) or 7.2 μ M and 15 μ M SM. The expression levels were normalized to the cell number via DRAQ5 staining and HaCaT control was set to 100 %. While Nrf2 expression is comparable under basal conditions, after SM treatment significantly lower levels (indicated by capped line) were detected in SM resistant cells. The induction of Nrf2 expression by SM in a dose-dependent manner could be shown in both cell lines.

Figure 5: Differences in expression levels of three different enzymes involved in glutathione cycle. For all In Cell Westerns, HaCaT (white) and HaCaT/SM (black) were exposed to 1.8 μM , 3.6 μM or 7.2 μM SM as well as EtOH as control (0 μM). Expression levels of glutamate-cysteine ligase (GCLC), glutathione S-transferase (GSTP1) and glutathione reductase (GSR) are shown normalized to cell number determined by DRAQ5. HaCaT control was normalized to 100 %. Results are shown as mean $\pm$ SD of triplicates and significance is indicated by a capped line.

Figure 6: Influence of glutathione S-transferase (GST) inhibitors and activators on cell survival after SM treatment.

Resistance against SM was tested for exposure with 0.2 to 500 μM SM for 5 days via cell survival (XTT) assay. Additionally, glutathione S-transferase (GST) inhibitors ezatiostat (TLK199) and ethacrynic acid were simultaneously incubated at 400 nM and 600 nM final concentrations, respectively. The GST activator ethoxyquin was used as a 24 h pretreatment before SM exposure with 24 μM . Controls did not contain inhibitors or activator. A.) Cell survival of HaCaT cells treated with control (black), ezatiostat (dashed) and ethacrynic acid (dotted). IC_{50} values are $6.0 \pm 1.1 \mu\text{M}$ (control), $2.7 \pm 1.1 \mu\text{M}$ (ezatiostat, $p < 0.0001$) and $2.5 \pm 1.1 \mu\text{M}$ (ethacrynic acid, $p < 0.0001$). B.) Cell survival of HaCaT/SM cells treated with control (black), ezatiostat (dashed) and ethacrynic acid (dotted). IC_{50} values are $58.4 \pm 1.1 \mu\text{M}$ (control), $36.2 \pm 1.1 \mu\text{M}$ (ezatiostat, $p < 0.01$) and $40.2 \pm 1.1 \mu\text{M}$ (ethacrynic acid, $p < 0.05$). C.) Cell survival of HaCaT controls (black) with IC_{50} of $6.5 \pm 1.2 \mu\text{M}$ and treated with ethoxyquin (dotted) with IC_{50} of $7.9 \pm 1.3 \mu\text{M}$ ($p > 0.05$). D.) Cell survival of HaCaT/SM treated with control (black) with IC_{50} of $34.5 \pm 1.1 \mu\text{M}$ and ethoxyquin (dotted) with $51.5 \pm 1.1 \mu\text{M}$ ($p > 0.05$).

Figure 7: Differences in expression levels of three different multidrug resistance (MDR) transporters.

HaCaT (white) and HaCaT/SM (black) cells were exposed to 1.8 μ M, 3.6 μ M or 7.2 μ M SM as well as EtOH as control (0 μ M) and expression levels were determined by In Cell Westerns. Expression levels are shown as mean $\pm$ SD of triplicates normalized to cell number via DRAQ5 and HaCaT control set to 100 %. A) Expression of P-glycoprotein (MDR1), B) expression of MDR3 and C) expression of MDR5.

Figure 8: Differences in intracellular NAD(H) level between SM-resistant and -sensitive cell lines. The levels of NAD⁺, NADH, NAD⁺/NADH ratio and total NAD were determined using NAD/NADH Quantification Kit (Sigma). Therefore, cells were exposed to 1.8 μM , 3.6 μM or 7.2 μM SM as well as EtOH as control (0 μM) for 24 hours. Afterwards, the assay was performed according to user's manual. The results are shown for HaCaT (white) and HaCaT/SM (black) as mean $\pm$ SD of triplicates. Significant differences ($p < 0.05$) are indicated by the capped line.

Figure 9: Differences in intracellular NADP(H) level between SM-resistant and -sensitive cell lines.

The levels of NADP⁺, NADPH, NADP⁺/NADPH ratio and total NADP were determined using NADP/NADPH Quantification Kit (Sigma). Therefore, cells were exposed to 1.8 μM, 3.6 μM or 7.2 μM SM as well as EtOH as control (0 μM) for 24 hours. Afterwards, the assay was performed according to user's manual. The results are shown for HaCaT (white) and HaCaT/SM (black) as mean ± SD of triplicates. Significant differences (p < 0.05) are indicated by the capped line.