Sulfur and nitrogen mustards induce characteristic poly(ADP-ribosyl)ation responses in HaCaT keratinocytes with distinctive cellular consequences

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HIGHLIGHTS

- Detailed analysis of sulfur and nitrogen mustard induced PARylation in HaCaT cells
- PARylation participates in mustard induced stress response in a substance specific manner
- PARP inhibitors potentiate CEES induced genome instability
- With limitations, CEES and HN2 can be used as surrogates to study SM related toxicity
- PARP inhibitors should be considered with caution as SM antidotes, due to potential long term effects

GRAPHICAL ABSTRACT

ABSTRACT

Mustard agents are potent DNA alkylating agents with mutagenic, cytotoxic and vesicant properties. They include bi functional agents, such as sulfur mustard (SM) or nitrogen mustard (mustine, HN2), as well as mono functional agents, such as “half mustard” (CEES). Whereas SM has been used as a chemical warfare agent, several nitrogen mustard derivatives, such as chlorambucil and cyclophosphamide, are being used as established chemotherapeutics. Upon induction of specific forms of genotoxic stimuli, several poly (ADP ribose) polymerases (PARPs) synthesize the nucleic acid like biopolymer poly(ADP ribose) (PAR) by using NAD⁺ as a substrate. Previously, it was shown that SM triggers cellular poly(ADP ribosyl) ation (PARylation), but so far this phenomenon is poorly characterized. In view of the protective effects of PARP

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inhibitors, the latter have been proposed as a treatment option of SM exposed victims. In an accompanying article (Debiak et al., 2015), we have provided an optimized protocol for the analysis of the CEES induced PARylation response in HaCaT keratinocytes, which forms an experimental basis to further analyze mustard induced PARylation and its functional consequences, in general. Thus, in the present study, we performed a comprehensive characterization of the PARylation response in HaCaT cells after treatment with four different mustard agents, i.e., SM, CEES, HN2, and chlorambucil, on a qualitative, quantitative and functional level. In particular, we recorded substance specific as well as dose and time dependent PARylation responses using independent bioanalytical methods based on single cell immuno fluorescence microscopy and quantitative isotope dilution mass spectrometry. Furthermore, we analyzed if and how PARylation contributes to mustard induced toxicity by treating HaCaT cells with CEES, SM, and HN2 in combination with the clinically relevant PARP inhibitor ABT888. As evaluated by a novel immunofluorescence based protocol for the detection of N7 ETE guanine DNA adducts, the excision rate of CEES induced DNA adducts was not affected by PARP inhibition. Furthermore, while CEES induced moderate changes in cellular NAD⁺ levels, annexin V/PI flow cytometry analysis revealed that these changes did not affect CEES induced short term cytotoxicity 24 h after treatment. In contrast, PARP inhibition impaired cell proliferation and clonogenic survival, and potentiated micronuclei formation of HaCaT cells upon CEES treatment. Similarly, PARP inhibition affected clonogenic survival of cells treated with bi functional mustards such as SM and HN2. In conclusion, we demonstrate that PARylation plays a functional role in mustard induced cellular stress response with substance specific differences. Since PARP inhibitors exhibit therapeutic potential to treat SM related pathologies and to sensitize cancer cells for mustard based chemotherapy, potential long term effects of PARP inhibition on genomic stability and carcinogenesis should be carefully considered when pursuing such a strategy.

1. Introduction

Sulfur mustard (SM), bis(β chloroethyl) sulfide also known as mustard gas, was first used in chemical warfare during WWI and later on in several other military conflicts and still remains a significant threat through its potential use in terrorist attacks or exposure of personnel involved in the disposal of old SM depots (Debiak et al., 2009; Mangerich and Esser, 2014). SM is a bi functional alkylating agent that reacts with many cellular targets, including lipids, proteins and nucleic acids, forming both intra and inter molecular cross links. It has strong vesicant and blister forming properties affecting primarily the skin, the eyes, and the respiratory tract. In particular, basal keratinocytes are sensitive to SM induced cytotoxicity causing detachment of these cells from the basement membrane and detachment of the epidermis from the dermis. Chronic toxicity comprises multiple skin and respiratory pathologies and an increased cancer risk (Kehe et al., 2009). Several clinical approaches have been tested for the development of antidotes to treat SM exposed people, such as antioxidants, scavengers, anti inflammatory drugs and protease inhibitors, but thus far, none of them yielded a satisfying therapy (Debiak et al., 2009; Kehe et al., 2009). Based on its commercial availability and access, the SM mono chloro derivative 2 chloroethyl ethyl sulfide (CEES) is widely used in laboratory settings as a SM substitute in experimental research. This compound also displays vesicant properties, but at ~ 100 fold higher concentrations (Wang et al., 2012). Ironically, in the aftermath of WWI and WWII, it was recognized that nitrogen mustard gas exerts strong cytostatic effects on the hematopoietic system. This led to the dawn of modern cancer chemotherapy through the synthesis of several nitrogen mustards, yielding the first chemotherapeutic drug mustine (HN2, bis[2 chloroethyl]methylamine) (Gilman and Phillips, 1946). When applied to skin, nitrogen mustards cause similar toxic effects and pathologies as compared to SM treatment (Tewari Singh et al., 2013, 2014b). Several nitrogen mustard based cytostatic compounds were developed, including chlorambucil, melphalan, and cyclophosphamide, all of which are still in clinical use today to treat various forms of leukemia and other malignancies (Neidle and Thurston, 2005).

At present the exact cellular mechanisms of mustard induced toxicity remain elusive, but it is thought that mustards elicit their toxic effects by induction of various forms of cell death and a strong

inflammatory response, primarily caused by unrepaired DNA damage (Kehe et al., 2009; Shakarjian et al., 2010). When SM reacts with DNA this mainly leads to the alkylation at the N7 position of guanine and at the N3 position of adenine resulting in the formation of N7 hydroxyethylthioethyl guanine (N7 HETE Gua) and N3 hydroxyethylthioethyl adenine (N3 HETE Ade), respectively. In addition, bi functional mustards, such as SM, HN2, or chlorambucil form biadducts between two guanine N7 positions to yield bis(N7guanine ethyl) sulfide (N7 Gua ETE N7 Gua) DNA crosslinks. Quantitative analysis estimated the relative distribution of these adducts to be ~ 60 88% dG monoadducts, ~ 10 42% dG crosslinks, and ~ 1 10% dA monoadducts depending on the methods used, the target material/tissue exposed, and the dose applied (Batal et al., 2013; Fidler et al., 1994; Yue et al., 2014). Crosslinks can be detected as early as 30 min after exposure in human lymphocytes (Debiak et al., 2011) and persist in SM exposed mouse tissue for up to several weeks (Batal et al., 2013). Interestingly, after cutaneous exposure, SM induced DNA adducts could also be detected systemically in many internal organs, such as brain, lung, kidney, and spleen (Batal et al., 2014). In contrast to bi functional agents, mono functional agents, such as CEES, can only form monoadducts, with N7 ethylthioethyl guanine (N7 ETE Gua) being the most abundant base damage, however still sufficient to induce genomic instability in human cells (Batal et al., 2014; Bennett et al., 2014). In addition, it has been reported that several hours after treatment, mustard agents induce oxidative stress via mitochondrial dysfunction, induction of ROS/RNS generating genes, and GSH depletion on a cellular level or via the recruitment of ROS/RNS generating immune cells on a tissue level, which also leads to the generation of oxidative DNA damage (Inturi et al., 2011; Jain et al., 2011; Steinritz et al., 2014). While mustards are not thought to produce DNA strand breaks by direct reaction with DNA, both DNA single as well as double strand breaks are introduced during subsequent DNA repair processes and potentially by secondary ROS formation (Debiak et al., 2011; Inturi et al., 2011; Jain et al., 2011). If persistent, mustard induced DNA damage can cause genetic mutations during cell division or induce cell death via apoptosis and necrosis (Boulware et al., 2012; Kehe et al., 2008; Matijasevic and Volkert, 2007; Powell et al., 2010).

Poly(ADP ribosyl) ation (PARylation) is a post translational modification that occurs under physiological and pathophysiological conditions and is involved in the genotoxic stress response. Poly

(ADP ribose) polymerases (PARPs, also named ARTDs) can bind to DNA single or double strand breaks and some other non canonical DNA structures, which triggers their enzymatic activation (Hot tiger, 2015). Activated PARPs use NAD⁺ as a substrate to synthesize the nucleic acid like biopolymer poly(ADP ribose) (PAR) with chain lengths of up to 200 ADP ribose units (Gibson and Kraus, 2012; Martello et al., 2013). PARylation refers to the covalent modification of proteins with PAR by modifying specific amino acids. Alternatively, a number of proteins can also noncovalently interact with PAR via distinct PAR binding motifs. By this means, PAR modulates physico chemical properties of target proteins including PARPs themselves and several factors involved in chromatin remodeling and DNA damage response (Krietsch et al., 2013). Importantly, DNA damage induced PARylation is transient and highly dynamic, since PAR is rapidly hydrolyzed by poly(ADP ribose) glycohydrolase (PARG) and other PAR degrading enzymes (Barkauskaite et al., 2013; Hottiger, 2015; Martello et al., 2013). PARylation exerts pleiotropic functions in genome maintenance, but also in a host of other cell functions such as chromatin remodeling, transcription, intra cellular signaling, cell cycle control, epigenetics and regulation of cell death (Beneke, 2012; Kraus and Hottiger, 2013; Robert et al., 2013). On an organismic level, these functions link PARylation to mechanisms of inflammation and metabolism as well as tumor suppression, for which PARP inhibitors are currently being tested in tumor therapy (Bai and Virag, 2012b; Curtin and Szabo, 2013; Mangerich and Bürkle, 2011, 2012).

Papirmeister et al. (1985) were the first to propose a biochemical hypothesis on the mechanism of SM induced cytotoxicity by suggesting that DNA strand breaks that are formed during cellular DNA repair activate PARPs to produce PAR by the consumption of cellular NAD⁺, thereby driving the cell into necrosis. Indeed several subsequent studies demonstrated a decline in intracellular NAD⁺ dependent on time and dose of SM exposure. This effect could at least partially be reversed by pharmacological PARP inhibitors suggesting a role for PARylation in SM induced NAD⁺ decreases (Hinshaw et al., 1999; Meier et al., 1987; Mol et al., 1989; Smith et al., 1990). However, if and how a PARP dependent decline in intracellular NAD⁺ levels contributes to SM induced cell death is as yet unclear: Several studies revealed a time dependent protective effect of PARP inhibitors on SM induced cytotoxicity (Meier, 1996; Meier and Johnson, 1992; Meier et al., 2000). It has to be noted that cytotoxicity in these studies was measured by dye exclusion and by this method only necrotic cells are scored as dead cells. Others did not observe such an effect (Kehe et al., 2008; Lin et al., 1994; Rosenthal et al., 2001; Smith et al., 1990), or even showed a sensitization of CEES induced cell death in the presence of PARP inhibitor treatment (Jowsey et al., 2009). Furthermore, it has been suggested that PARP inhibition induces a switch in the mode of cell death by inhibiting necrosis but driving cells into apoptosis (Kehe et al., 2008; Meier and Millard, 1998; Rosenthal et al., 2001). Rosenthal et al. reported that such an effect may even be cell type specific, as it was only observed in skin fibroblasts, but not in keratinocytes (Rosenthal et al., 2001). Based on the rationale that PARP inhibition preserves NAD⁺ levels and counteracts inflammation, PARP inhibitors were tested as potential therapeutic agents for SM exposures. Interestingly, in several animal models PARP inhibition and/or boosting of NAD⁺ synthesis led to reduced pathological signs upon SM exposure (Cowan et al., 2003; Gross et al., 1985; Mol et al., 1991; Yourick et al., 1991, 1993; Zhang et al., 1995).

Despite previous reports on the effect of PARP inhibitors on SM induced NAD⁺ depletion, cell death and pathology, specific reports characterizing the actual PARylation response upon mustard treatment are incomplete and inconsistent. Using an immunochemical approach, Rosenthal et al. (1998) analyzed PAR formation

in keratinocytes upon treatment with a single dose SM (100 µM) from 2 to 24 h, revealing auto modified PARP1 peaking 2 h after treatment. Using ³H NAD⁺ as a substrate, Hinshaw et al. (1999) analyzed PAR formation in permeabilized endothelial cells and keratinocytes upon treatment with 250 and 500 µM of SM for 2, 3, and 5 h, showing a 2–4 fold increase in signal intensities (Hinshaw et al., 1999). Using fluorescence microscopy, Seker et al. (2000) showed PAR formation in MCF7 cells 24 h after glufosfamide treatment (50–750 µM). Using biotinylated NAD⁺ in an ELISA approach, Kehe et al. assessed PAR formation in permeabilized HaCaT cells upon pulse treatment of cells for 30 min with 10–1000 µM SM followed by further incubation of cells for 45 min in SM free medium leading to a maximum four fold increase in signal intensities (Kehe et al., 2008). Finally, also using biotinylated NAD⁺, Paramov et al. (2011a) assessed PARP activity in HaCaT cell extracts upon treatment of cells for 1 h with 0.5–5 mM CEES, leading to a maximum of a ~10 fold in signal intensities at 5 mM CEES treatment.

Even though the above cited literature suggests a role for PARylation in mustard induced toxicity, so far, a systematic and comprehensive analysis of PARylation in response to chemical mustard exposure and its role during subsequent genotoxic stress response is not available. However, a detailed characterization of such mechanisms would help to clarify if, on the one hand, PARP inhibitors may potentiate carcinogenic effects of mustard exposure when applied as anti inflammatory antidotes to SM exposed subjects; or, on the other hand, if PARP inhibitors may be used as chemosensitizers in nitrogen mustard based cancer therapy. In an accompanying article in this special issue, we have established and validated a protocol to analyze CEES induced PAR formation in HaCaT cells by means of single cell immunofluorescence microscopy. Moreover, we provided a detailed analysis of the CEES induced PARylation response in HaCaT keratinocytes, which forms an experimental basis to study the molecular mechanism of PARP activation after mustard treatment (Debiak et al., 2015). In the present study, we investigated PARylation responses and functional consequences thereof in human HaCaT keratinocytes after treatment with a panel of mono- and bi functional mustard agents, i.e., SM, CEES, HN2, and chlorambucil.

2. Materials and methods

2.1. Laboratory safety

Mustards are highly toxic and carcinogenic substances. The use of some of them, i.e., SM is highly restricted and its use in experimental studies requires a special license. All of those substances must be handled with utmost caution, in an appropriate laboratory setting by experienced personnel with adequate protection. All experiments with SM of the present study were performed at the Bundeswehr Institute of Pharmacology and Toxicology, Munich, Germany.

2.2. Treatment of HaCaT cells with chemicals

The human keratinocyte cell line HaCaT (Boukamp et al., 1988) was cultured in DMEM (Life Technologies) containing 10% fetal calf serum (PAA Laboratories) and 1% penicillin streptomycin (Life Technologies) at 37 °C, in a humidified incubator with 5% CO₂. The pharmacological compound ABT888 (Selleckchem) was used to inhibit PARP activity. Stock solutions of ABT888 (2.5 mM) were prepared in ddH₂O and stored at –80 °C. Cells were supplemented with 1 µM ABT888 in fresh medium 30 min prior to mustard treatment (or as indicated). Premixes (100×) of SM or CEES, or HN2 solutions were prepared in EtOH (95% v/v)/HCl (0.5% v/v) as described in Debiak et al. (2015). Briefly, directly before cell

treatment, 1 × solutions (1% v/v, final concentrations as indicated) were prepared in pre warmed PBS (37 °C) and immediately added to the cells. Equal distribution of mustard agents in PBS was ensured by rigorously mixing the tubes. If not indicated otherwise, controls were incubated with 1% (v/v) EtOH/HCl in PBS.

2.3. Analysis of PAR formation by isotope dilution mass spectrometry

Analysis of cellular PAR levels by mass spectrometry were performed as described previously (Martello et al., 2013). Briefly, in order to extract cellular PAR, treated cells were rinsed with PBS (pH 7.4) and harvested by addition of 2 ml 20% TCA (w/v) and the acid insoluble material was detached from the dishes by scraping with a rubber policeman. Subsequently, pellets were washed with ice cold 70% ethanol, resuspended in 225 µl 0.5 M KOH, 50 mM EDTA and incubated at 37 °C for 45 min. Alkaline treatment was stopped by neutralization, and 5 pmol of ¹³C, ¹⁵N labeled PAR was added as an internal standard. DNA and RNA were digested by addition of 44 mM MgCl₂, 0.5 mM CaCl₂, (0.1 mg/ml) DNase I (Roche) and 55 µg/ml RNase A (Sigma Aldrich) for 3 h at 37 °C, following digestion with 0.2 mg/ml proteinase K (Roche) overnight. PAR extractions were performed using the High pure miRNA isolation kit (Roche) according to the manufacturer's instructions. In order to prepare samples for LC MS/MS analysis, PAR was subjected to digestion with alkaline phosphatase from bovine intestine mucosa (AP) (Sigma Aldrich) and phosphodiesterase I (PDE) (Affymetrix) before LC MS/MS analysis. Samples were brought to a final volume of 500 µl in a solution containing 1 mM MgAc, 30 mM NH₄Ac, 10 U AP and 0.5 U PDE. Following incubation for 3 h at 37 °C, the enzymes were removed using a 10 kD cut off filter (nanosep 10K, Pall) by centrifugation for 20 min at 14,000 × g. Cell extracts were vacuum dried and resuspended in 100 µl of mobile phase prior to LC MS/MS analysis. HPLC separation of digestion products was performed using a Waters 2695 separation module and a Hypersil Gold aQ 150 × 2.1 mm particle size 3 micron (Thermo Scientific) at 30 °C. Molecules were separated isocratically with 1% acetonitrile supplemented with 0.1% acetic acid at a flow rate of 0.3 ml/min. The column effluent was coupled to a Quattro Micro mass spectrometer (Waters) operating in positive ESI mode using parameters as reported previously (Martello et al., 2013).

2.4. Analysis of PARYlation by immunofluorescence microscopy

Immuno chemical analysis of PAR formation was performed as described in Debiak et al. (2015) with some modifications. Cover slips were placed into 12 well plates and 3.0 × 10⁵ cells/well were seeded and incubated for 24 h prior treatment. Cells were pre incubated with ABT888 for 30 min (or as indicated) and treated with doses of SM/CEES/HN2/chlorambucil for times as indicated at 37 °C. Controls were treated with 0.1 mM H₂O₂ for 5 min. Negative controls were incubated with EtOH/HCl in PBS (EtOH/solvent control). Cells were washed in ice cold PBS and fixed in ice cold 100% methanol for 7 min. Then, cover slips were washed three times with PBS for 5 min, blocked in TBS containing 5% (w/v) skim milk powder and 0.1% (w/v) Tween20 for 30 min at 37 °C, and samples were incubated with anti PAR primary antibody (10H) over night at 4 °C in a humidified chamber. Cover slips were washed three times for 5 min in PBS and incubated with an Alexa488 labeled secondary antibody for 1 h at 37 °C in the dark in a humidified chamber. Cover slips were washed three times for 5 min in PBS and incubated with Hoechst 33342 (0.2 µg/ml in PBS) for 5 min at RT, washed again three times for 1 min in PBS, and mounted on glass slides using Aqua PolyMount (Polysciences). Images were acquired using a Zeiss Axiovert 200 M epifluorescence microscope equipped with an AxioCam MRm camera, and were analyzed with the Axiovision or ImageJ software. Imaging data

were also analysed by TissueFAXS Software (3.5.5120.128) and TissueQuest Software (4.0.1.0127) (TissueGnostics). Laser scanning microscopy pictures were acquired with a Zeiss LSM 710 microscope.

2.5. Analysis of N7 (H)ETE guanine adducts by immunofluorescence microscopy

For immunofluorescence staining for mustard induced guanine adducts, cells on cover slips were washed twice with PBS and were fixed with methanol containing 3% acetic acid at 20 °C. Then, slides were washed with PBS four times for 5 min, cells were permeabilized in 0.1% sodium citrate and 0.3% Triton X100 for 10 min at 4 °C, cultures were washed again with PBS four times, and were incubated in 80% formamide in PBS for 30 min at 75 °C. Subsequently, plates were incubated for 5 min at 4 °C and then with the N7 (H)ETE guanine specific primary mouse monoclonal antibody 2F8 (TNO, The Netherlands) (Kehe et al., 2013) diluted 1:5000 in Dako Antibody Diluent at 4 °C overnight. Then, samples were washed four times for 5 min and incubated with an Alexa488 coupled secondary antibody diluted in Dako Antibody Diluent for 90 min at 37 °C. Finally, samples were washed four times, DNA was stained with Hoechst 33,342 (0.2 µg/ml in PBS) for 5 min at RT, and cover slips were mounted on glass slides using Aqua PolyMount (Polysciences). Images were acquired using a Zeiss Axiovert 200 M epifluorescence microscope equipped with an AxioCam MRm camera, and were analyzed with Axiovision or ImageJ software.

2.6. Analysis γ H2A.X formation by immunofluorescence microscopy

For detection of γ H2A.X an immunocytochemistry staining was performed. Cells were fixed with 4% PFA at RT for 30 min. For permeabilization, cells were incubated in PBS with 0.1% sodium citrate and 0.3% Triton X100 for 10 min at 4 °C. After several washing steps, unspecific binding sites were saturated with blocking solution (DakoCytomation) for 30 min at RT. Then samples were incubated with primary antibody solution (Millipore, 1:600) over night at 4 °C. The secondary antibody Dy549 anti mouse (Vector, 1:800) was incubated for 1.5 h at RT. All steps were performed in an automated manner by using a pipetting robot with temperature control (Tecan freedom evo). Nuclei were stained with DAPI by incubating samples for 5 min with 300 nM DAPI (BioLegend). After several washing steps, cells were then embedded with ProLongAntifade (Invitrogen). The fluorescence intensity in all nuclei was analyzed by a microscope system of TissueGnostics using TissueQuest software.

2.7. Determination of LC₅₀ values of alkylating compounds

HaCaT cells were seeded in 96 well plates at a density of 25,000 cells per well and grown in DMEM, supplemented with 10% FCS for 24 h. DMEM was removed and cells were exposed to a specific agent, either SM, HN 2, chlorambucil or CEES at concentrations of 12.4 µM, 37.0 µM, 111 µM, 333 µM, 1000 µM and 3000 µM for 1 h. In case of SM, the 3000 µM concentration was omitted and concentrations from 4.1 µM to 1000 µM were used. Agents used for exposure were dissolved in Minimum Essential Medium (MEM). Control wells received pure MEM, whereas 100% cytotoxicity was induced by 0.1% Triton X 100, dissolved in PBS. Each exposure group consisted of 12 wells, i.e. a row on the 96 well plate. After 1 h, poisoned and control medium was removed and replaced by fresh DMEM, supplemented with 5% FCS. Cells were incubated for 48 h. Cell vitality was then determined, using the XTT assay (Roche, Switzerland). Non linear regression of dose response curves and determination of LC₅₀ values was conducted, using GraphPad PRISM software.

2.8. Annexin V/propidium iodide staining

To analyze the mode of cell death after CEES treatment, HaCaT cells were seeded at 6.0×10^5 cells/well in 12 well plates and incubated for 24 h. Prior treatment of cells with CEES for 1 h, cells were pre incubated with ABT888 for 30 min. Then, cells were washed with PBS, supplemented with medium and incubated for another 23 h. Thereafter, medium containing cell debris and detached cells were collected in pre chilled 15 ml tubes. Cells were trypsinized, added to the tubes as well, all cells were pelleted at 1000 rpm for 5 min at 4 °C, washed with 5 ml cold PBS, and counted with a CASY cell counter (Roche). Then, cells were centrifuged at 1000 rpm for 5 min at 4 °C and resuspended in annexin V binding buffer at a final titer of 2.0×10^6 cells/ml. In the dark, 2.5 µl of annexin V FITC solution (Enzo Lifesciences) was mixed with 50 µl cell suspension, samples were incubated for 15 min on ice, and 245 µl annexin binding buffer (10 mM HEPES/NaOH, pH 7.4, 140 mM NaCl, 2.5 mM CaCl_2) and 1 µl propidium iodide (1.0 mg/ml) were added. Subsequently, samples were subjected to flow cytometric analysis (BD LSR II).

2.9. NAD^+ cycling assay

NAD^+ levels were determined as described previously using an enzymatic cycling assay modified from (Jacobson and Jacobson, 1976). Briefly, after treatment, cells were harvested and 1×10^6 cells were resuspended in 1 ml PBS and immediately placed on ice. Subsequently, 48 µl of perchloric acid (11.63 M) was added, reaction mixtures were incubated for 15 min on ice, and centrifuged for 10 min at $1500 \times g$. Then, the supernatant was

mixed with 700 µl of 0.33 M K_2HPO_4 (pH 7.5), centrifuged, snap frozen in liquid nitrogen and stored at -80 °C. After thawing and centrifugation, 40 µl samples were mixed with 160 µl of buffer A (0.25 M H_3PO_4 , 0.5 M NaOH) and 100 µl of buffer B [0.34 M bicine NaOH (pH 8.0), 2.9 mg/ml BSA, 14.3 mM EDTA, 1.4 mM MTT, 1.7 M EtOH, 5.7 mM phenazine ethosulfate, 0.14 mg/ml ADH]. After a 30 min incubation period at 30 °C, absorption was measured at 550 nm (with 690 nm as a reference wavelength) in a 96 well plate ELISA reader.

2.10. Proliferation assay

Cells were seeded in 12 well plates in triplicates and treated with ABT888 and CEES as described above. For time series experiments, non treatment controls were included for every time point. At the time points of interest, cells were trypsinized and cell numbers were determined using a CASY cell counter (Roche).

2.11. Cytokinesis block micronucleus assay

The assay was performed based on a protocol published by (Fenech, 2007) with some modifications. Briefly, 3×10^5 cells were seeded on cover slips and incubated in medium supplemented or not with 1 µM ABT888, 30 min prior to mustard exposure for 1 h. Thereafter, samples were washed twice with PBS and cells were incubated for another 24 h in medium containing 6 µg/ml cytochalasin B (Sigma Aldrich) to block cytokinesis in the absence or presence of ABT888. After 24 h, supernatant was removed and slides were washed twice and fixed with pre cooled methanol at 20 °C for 7 min. Subsequently, samples were washed three times

Fig. 1. Mustard agents and their cytotoxicity profiles. **A.** Chemical structures of sulfur and nitrogen mustards as used in this study. CEES is a mono-functional agent, whereas sulfur mustard, HN2, and chlorambucil are bi-functional agents. **B.** Cytotoxicity profiles and LC50 values of substances as shown in A. HaCaT cells were treated with chemicals in concentrations as indicated and subjected to cytotoxicity testing 48 h post treatment. Data represent means $\pm$ SD from 6–7 independent experiments.

Fig. 2. Sulfur and nitrogen mustards induce dose-dependent PAR formation in HaCaT cells. **A.** Dose response relationship of HN2 treatment and PAR formation as evaluated by immunofluorescence microscopy. Cells were treated with HN2 at concentrations as indicated for 20 min or with 100 μ M H₂O₂ for 5 min as positive control and subjected to immunofluorescence staining. Scale bar indicates 20 μ m. Densitometric evaluation of microscopic data; means $\pm$ SD of 3 independent experiments. **C.** Characterization of H₂O₂-, CEES- and SM-induced PARYlation in HaCaT cells using laser scanning microscopy. Cells were treated with 3 mM CEES and 0.3 mM SM for 10 min or with 1 mM H₂O₂ for 5 min. **D.** Dose-dependent induction of PAR formation after treatment with low doses of SM, CEES, and HN2 as analyzed by quantitative isotope-dilution mass spectrometry as described in (Martello et al., 2013). Data represent means $\pm$ SD from at least 3 independent experiments. Statistical analysis was performed using one-way ANOVA testing followed by Dunnett's multiple comparison testing. * P < 0.05, ** P < 0.01; *** P < 0.001.

for 5 min, DNA was stained with Hoechst33342, and coverslips mounted on glass slides using Aqua PolyMount (Polysciences). Counting of micronuclei was performed by a blinded evaluator using a Zeiss Axiovert 200 M epifluorescence microscope following the criteria by Fenech (Fenech, 2007).

2.12. Clonogenic survival assay

The assay was performed based on a protocol published by Franken and colleagues (Franken et al., 2006) with some modifications. Briefly, HaCaT cells (2×10^5 cells/ml) were

incubated for 10 min in the presence or absence of 1 μ M ABT888 before addition of 10 μ l of a 100 $\times$ CEES stock or EtOH/HCl control solutions. After 30 min incubation, 100 μ l of the cell suspensions were transferred into 9.9 ml of PBS, resulting in a cell titer of 2 cells/ μ l. Cells were seeded in adequate cell numbers in 6 cm plates and incubated for 2 weeks in the presence or absence of 1 μ M ABT888. Then, medium was removed and colonies were fixed and stained for 30 min using a solution of 6% glutaraldehyde (Sigma Aldrich) mixed with 0.5% crystal violet. The culture dishes were washed with PBS and colonies consisting of at least 50 cells were counted using a stereomicroscope (Leica).

Fig. 3. Mustards induce a highly dynamic and substance-specific PARylation responses. A. Time-dependent cellular PARylation in HaCaT cells after treatment with mustards as analyzed by epi-immunofluorescence microscopy. Cells were treated with SM, CEES, and HN2, fixed at time points as indicated, and stained for cellular PAR using an anti-PAR-specific mAb (10H). H₂O₂ (100 μ M) treatment for 5 min served as a positive control. Scale bars indicate 50 μ m. B. Signal intensities of individual cells were densitometrically evaluated. Data represent means $\pm$ SD. Statistical analysis was performed using one-way ANOVA testing followed by Dunnett's multiple comparison testing. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

2.13. Statistical analysis

All experiments were performed in replicate numbers as indicated and data analyzed with GraphPad Prism Software, using statistical tests as indicated.

3. Results

3.1. Mustards exhibit substance specific cytotoxicity profiles

Epidermal keratinocytes are considered one of the primary targets for mustard induced pathologies (Kehe et al., 2009). In the present study, we used HaCaT cells as an established human keratinocyte model (Boukamp et al., 1988) to study the PARylation dependent molecular toxicology of mustards. To define LC50 values of mustards in these cells and under experimental conditions as used in this study, we performed a comparative cytotoxicity study on a panel of mustard agents, including mono and bi functional nitrogen as well as sulfur mustards (Fig. 1). As is evident from cytotoxicity profiles, the bi functional mustards SM and HN2 exhibit the lowest LC50 values ($\sim 100 \mu\text{M}$) whereas the pharmacologically relevant bi functional nitrogen mustard chlorambucil displayed an LC50 value in the sub millimolar range ($377 \mu\text{M}$) and the mono functional half mustard CEES at concentrations $> 1 \text{ mM}$. Based on the finding of similar cytotoxicity of SM and HN2 as well as the structural similarity of SM with CEES, we selected SM and its analogues HN2 and CEES as model substances for further in depth analysis. However, one needs to keep in mind

that the CEES toxicity depends on specific conditions of the treatment protocol. The studies of CEES induced cytotoxicity in HaCaT cells reported LC50 values differing by at least one order of magnitude within a range of 0.25 to 2 mM depending on treatment time and conditions applied (Paromov et al., 2011a,b; Tewari Singh et al., 2010, 2014a).

3.2. Sulfur and nitrogen mustards induce PARylation in HaCaT cells in a dose and time dependent manner

Despite the existence of sporadic reports of mustard induced cellular PARylation response (see above, Section 1), this phenomenon is poorly characterized. In an accompanying article (Debiak et al., 2015), we established a reliable protocol for the treatment of HaCaT cells with CEES and subsequent analysis of cellular PARylation response using immunofluorescence microscopy. Here we applied this protocol to examine systematically dose and time dependent PARylation dynamics upon treatment of HaCaT cells with different bi functional mustard derivatives (SM, HN2, and chlorambucil). We analyzed cellular PAR formation using two completely independent bioanalytical approaches (Figs. 2 and 3). Our data obtained with semi quantitative single cell immunofluorescence microscopy using the PAR specific antibody 10H revealed that both nitrogen and sulfur mustards induce cellular PAR formation in living cells in a dose dependent manner (Figs. 2 and 3). As expected, mustard agents induced PAR formation at a lower level than the oxidizing genotoxic agent and strong PARP activator H_2O_2 , used as a positive control (Fig. 2A C). We used

Fig. 4. Dose- and time-dependent chlorambucil-induced PARylation in HaCaT cells. A. Dose response relationship. Cells were treated with chlorambucil at concentrations as indicated for 90 min and subjected to immunofluorescence staining. B. Time-dependent cellular PARylation after treatment with chlorambucil (2 mM) for times as indicated followed by immunofluorescence staining. H_2O_2 (100 μM) treatment for 5 min served as a positive control. Scale bars indicate 20 μm .

Fig. 5. Analysis of short-term cytotoxicity, NAD⁺ levels, and cell proliferation after combined treatment of HaCaT cells with CEES and PARP inhibitor ABT888. **A** and **B.** CEES treatment induces a moderate decrease in intracellular NAD⁺ levels in HaCaT cells that can be rescued by PARP inhibitor treatment. Cells were pre-incubated ± ABT888 (1 μM) for 30 min and then treated with genotoxins as indicated. **A.** Dose-response relationship of CEES treatment and intracellular NAD⁺ levels. Cells were treated with CEES at concentrations as indicated for 10 min. H₂O₂ (1 mM) treatment served as a positive control. Control indicates EtOH/HCl solvent control. Data represent means ± SD from 3 independent experiments. **B.** Time-course analysis of intra-cellular NAD⁺ levels after low-dose CEES treatment (250 μM) for 1 h. Data represent means ± SD from >3 independent experiments. **C.** CEES-induced short-term cytotoxicity does not depend on cellular PARylation. Cells were pre-incubated ± ABT888 (1 μM) for 30 min, treated with CEES and H₂O₂ at concentrations as indicated for 1 h, and subjected to cell death analysis 23 h later using Annexin V/propidium iodide staining and flow cytometric analysis. Data represent means ± SEM from >3 independent experiments. Viable cells refer to annexin V/PI-double negative cells; apoptotic cells to annexin V-positive, PI-negative cells; and necrotic and late-apoptotic cells to annexin V/PI-double positive cells (ratios compared to total cell numbers). **D** and **E.** PARP inhibition

confocal microscopy to characterize accurately the intra cellular distribution of PAR in HaCaT cells upon mustard treatment. SM, CEES and H_2O_2 treatment led to exclusively nuclear PAR formation at distinct foci, strongly indicating an active role of PARylation in mustard induced genotoxic stress response. (Fig. 2C). Analysis of cellular PAR formation upon low dose mustard treatment using quantitative isotope dilution mass spectrometry as published recently (Martello et al., 2013) proved the dose dependent formation of PAR molecules upon mustard treatment with unequivocal chemical specificity (Fig. 2D). Specifically, these experiments revealed that bi functional mustards, i.e., SM and HN2, are able to induce cellular PARylation in the low to medium micromolar range, whereas millimolar concentrations of the mono functional agent CEES are necessary to induce PARylation significantly. This is consistent with the fact that CEES induced cytotoxicity is >10 fold lower than those of SM and HN2 (Fig. 1). Interestingly, PARylation dynamics considerably differed between bi and mono functional mustard derivatives, indicating major differences in the toxicokinetic and/or toxicodynamic properties of these compounds (Fig. 3). After CEES treatment, PAR formation peaked at 10 min post treatment and declined close to the threshold of sensitivity after 45 min. No second wave of PARylation was observed up to 6 h after treatment (Fig. S1). However, it is important to note that even 6 h after treatment, PAR signal intensity was above background, indicating sustained low level stimulation of PARP activity (Fig. S1). In contrast, the bi functional compounds clearly induced a biphasic PARylation response, showing a moderate first wave of PARP activation within the first 2–10 min, which declined close to the threshold of sensitivity after 30 min, before inducing a second wave of PARylation with peak values after 60 min. Of note, the pharmacologically relevant bi functional compound chlorambucil also induced PAR formation in HaCaT cells with similar dynamics as observed for SM and HN2 (Fig. 4), suggesting a role for mustard induced PARylation in chlorambucil related cancer therapy.

As shown in Debiak et al. (2015), when cells were pre incubated for 30 min with 1 μ M of the PARP inhibitor ABT888, PAR formation could be strongly inhibited after genotoxic treatment with CEES or H_2O_2 . To this end, we used this protocol to explore functional consequences of mustard induced PAR formation as reported in the following sections.

3.3. Analysis of mustard induced short and long term cytotoxicity as a function of PARP activity

Previously it was reported that SM treatment led to a lowering of NAD^+ levels, an effect that in some studies could be inhibited by PARP inhibitor treatment (see above, Section 1). While Kehe et al. (2008) observed no changes in overall cytotoxicity upon SM and PARP inhibitor treatment, their results suggested that PARP inhibition induces a switch in the mode of cell death by inhibition of necrosis but promotion of apoptosis. To test if the same holds true upon CEES treatment we exposed HaCaT cells to increasing doses of CEES and determined intra cellular NAD^+ levels using an enzymatic NAD^+ cycling assay (Jacobson and Jacobson, 1976). As expected, treatment of cells with 1 mM H_2O_2 for 10 min led to a massive lowering of NAD^+ levels, an effect that was almost completely abrogated by pre incubating cells with ABT888 (Fig. 5A). In contrast, short term treatment of HaCaT cells for 10 min with CEES in concentrations up to 3 mM reduced NAD^+ levels only by ~10%. Despite the unexpected finding that this

reduction showed no dose dependency, reduced NAD^+ levels were most likely caused by PARP activation, since the effect could be completely abrogated by ABT888 treatment. These findings are in line with time course experiments demonstrating that low dose CEES treatment (250 μ M) leads to a significant drop in NAD^+ levels by 40% at 3–6 h after treatment, followed by partial recovery at 24 h after treatment. Once again, application of ABT888 prevented the CEES induced lowering of NAD^+ levels (Fig. 5B). Next, to test if these moderate decreases in NAD^+ levels may affect cellular survival, we treated HaCaT cells with increasing concentrations of CEES and performed cytotoxicity analyses. As expected, annexin V in combination with propidium iodide staining revealed that PARP inhibition significantly reduced apoptotic and necrotic cell death upon H_2O_2 treatment, most likely due to inhibition of PARP1 and consequently preservation of NAD^+ levels (Fig. 5C). It is interesting to note that at lower concentrations of H_2O_2 treatment (up to ~3 mM) PARP inhibition significantly prevented apoptosis induction, while at higher concentrations of H_2O_2 treatment (>5 mM) inhibition of necrotic cell death became more prevalent. In line with the rather moderate reduction of NAD^+ levels upon CEES treatment, but contrary to previous reports on SM induced cytotoxicity, no effect of PARP inhibition on CEES induced cell death was observed at all, i.e. neither for apoptotic nor for necrotic cell death (Fig. 5C). To test if less drastic endpoints than cell death were affected by PARP inhibition, we determined the proliferation capacity of HaCaT cells upon CEES treatment in the absence or presence of ABT888. While ABT888 treatment alone slightly, but non significantly, affected cell proliferation, CEES treatment significantly impaired cell proliferation, as expected, at sub millimolar concentrations (Fig. 5D and E). Importantly, this effect was potentiated by ABT888. These results indicate that mustard induced PARylation indeed plays a functional role in cellular stress response. In order to characterize this further, we examined clonogenic survival of HaCaT cells upon mustard and PARP inhibitor treatment. The clonogenic survival assay is a very sensitive long term toxicity test, which apart from cell death, takes into account impaired cell adhesion and proliferation effects. Fig. 6 demonstrates that the sole treatment with SM, CEES, or HN2 led to dose dependent impairment of clonogenic survival with EC50 values for SM and HN2 in the low micromolar range, and for CEES in the high micromolar range. Interestingly, while ABT888 treatment alone did not affect clonogenic survival (Fig. 6B), it significantly sensitized cells to mustard induced toxicity for all three mustards tested, with the largest sensitization observed for SM treatment (Fig. 6).

3.4. Analysis of mustard induced genotoxicity as a function of PARP activity

A key question of this study was to analyze if PARP inhibition sensitizes HaCaT cells to mustard induced genotoxic effects. If this were the case it would imply that PARP inhibitors are contra indicated for SM exposed subjects, due to potential potentiation of SM induced carcinogenesis. To this end, we analyzed two endpoints of mustard induced genotoxicity, i.e., DNA adducts and micronuclei formation rates.

3.4.1. Excision of CEES induced guanine adducts is not affected by PARP inhibition

To test if PARylation already affects early stages of mustard induced DNA damage response, we analyzed if PARP inhibition

combined with CEES treatment impairs proliferation of HaCaT cells. Cells were pretreated $\pm$ ABT888 (1 μ M) for 30 min and then treated with CEES for 1 h. D. After CEES treatment (0.5 mM), samples were incubated for time points as indicated ($\pm$ ABT888) and then subjected to cell counting. The inhibitor alone did not influence the cell growth. Data represent means $\pm$ SEM from three replicates. E. After CEES treatment at concentrations as indicated, cells were incubated for 72 h ($\pm$ ABT888) and subjected to cell counting. Data represent means $\pm$ SEM from 3 independent experiments. Statistical evaluation was performed using two-way ANOVA testing followed by Sidak's multiple comparison testing. * P < 0.05, ** P < 0.01; *** P < 0.001.

Fig. 6. PARP inhibition combined with mustard treatment impairs clonogenic survival in a substance specific manner. Cells were pre-incubated $\pm$ ABT888 (1 μ M) for 10 min and then treated with mustards at concentrations as indicated for 30 min. After incubation for 2 weeks $\pm$ ABT888 (1 μ M), samples were subjected to clonogenic survival analysis. A. Representative experiment showing clonogenic survival of HaCaT cells after sulfur mustard treatment. B. ABT888 treatment alone did not significantly influence plating efficiency of HaCaT cells (not significant compared to 100% as evaluated by one-sample *t*-test). C-E. Dose-response curves for cells treated with SM (B), CEES (C), and HN2 (D). Data represent means $\pm$ SD from ≥ 3 independent experiments performed in technical triplicates. Statistical evaluation was performed using a paired two-way ANOVA test followed by Sidak's multiple comparison testing (* $P < 0.05$, ** $P < 0.01$; *** $P < 0.001$) or Student's *t*-test (** $P < 0.01$).

affects the excision rate of mustard induced guanine base damage. To this end, we modified a recently reported immuno slot blot protocol for the detection of SM and CEES induced N7 (H)ETE guanine base damage (Kehe et al., 2013) for usage in an immunofluorescence setting. Fig S2 demonstrates that this assay is able to detect CEES induced N7 ETE guanine adducts at a concentration of $>100 \mu\text{M}$ CEES and SM induced N7 HETE guanine adducts at a concentration of $>10 \mu\text{M}$. Using the same assay, we monitored the excision kinetics of damaged bases in HaCaT cells after a single dose treatment of CEES for the time course of 2 days. Interestingly, even two days after CEES treatment, N7 ETE guanine derived signals were clearly above background, indicating that not all base lesions are repaired during this repair period (Fig. 7). No significant effect of PARP inhibition on the removal N7 ETE guanine adducts was observed within 48 h after mustard treatment. This is consistent with the notion that PAR formation is induced by DNA strand breaks that occur during the repair of DNA adducts. Although this hypothesis needs to be analyzed in greater detail, γ H2A.X staining (Fig. S3) as well as previous reports using an automated DNA alkaline unwinding assay (i.e., FADU assay, (Debiak et al., 2011)) support the notion that DNA strand breaks are formed during SM induced genotoxic stress response, which can lead to PARP1 binding and activation.

3.4.2. PARP inhibition potentiates CEES induced micronuclei formation

To test if PARYlation affects later stages of the mustard induced genotoxic stress response we performed the cytokinesis block micronucleus assay. Micronucleus formation is a sensitive

biomarker for major genomic rearrangements that occur in dividing cells and is commonly used in genotoxicity testing (Fenech, 2007) (Fig. 8A). While mustard or ABT888 treatment alone did not significantly increase micronucleus frequencies in HaCaT cells, cultures treated with mustards in combination with ABT888 showed a moderate increase in micronucleus frequencies, which reached statistical significance in samples co treated with CEES and ABT888 (Fig. 8B and D). These results provide strong evidence for an active role of cellular PARYlation in mustard induced genotoxic stress response in order to protect and restore cellular function upon the genotoxic insult. In conclusion, our results show that the use of PARP inhibitors as countermeasures to treat mustard induced injuries must be carefully considered in view of risk benefit analysis and might be restricted to treatment of acute inflammatory conditions; however, they may raise the possibility to use PARP inhibitors as chemosensitizing agents for mustard based cancer therapy.

4. Discussion

In an accompanying article, we have established a treatment protocol and provided a detailed analysis of the CEES induced PARYlation response in HaCaT (Debiak et al. 2015). The present study provides now a systematic and comparative characterization of the mustard induced PARYlation responses and its functional consequences with respect to mustard induced cytotoxicity and genotoxicity in HaCaT cells after treatment with a panel of mono and bi functional mustard agents. Our data revealed substance specific, dose and time dependent PARYlation dynamics after

Fig. 7. Excision of CEES-induced N7-ETE-guanine adducts is not dependent on cellular PARYlation. **A.** Cells were pre-incubated $\pm$ ABT888 (1 μ M) for 30 min, treated with 1 mM CEES for 1 h, then incubated up to time points as indicated, and subjected to immunochemical staining using an N7-(H)ETE-G-specific antibody 2F8 as described in [Kehe et al., 2008](#). Scale bar indicates 50 μ m. **B.** Densitometric analysis from three independent experiments, means $\pm$ SD. Statistical evaluation was performed using two-way ANOVA testing followed by Sidak's multiple comparison testing. For a dose-response analysis of CEES-induced N7-ETE-guanine adducts refer to Fig. S2.

treatment of HaCaT cells with four different sulfur and nitrogen mustards using completely independent bioanalytical methods based on immunofluorescence microscopy and quantitative isotope dilution mass spectrometry. These results demonstrate that bi functional agents, such as SM and HN2 induce a biphasic PARYlation response with peak values after 60 min, whereas mono functional CEES induces only a single wave of PARYlation with much lower potency that peaks at 10–15 min. Furthermore, we provide evidence that PARYlation has functional consequences in mustard induced cellular stress response, since PARP inhibition sensitizes cells to mustard treatment in terms of clonogenic survival (SM, CEES, and HN2), cell proliferation (CEES), and micronucleus formation (CEES). These results have several important implications in terms of mechanisms of toxicity of nitrogen and sulfur mustards as well as towards the potential of PARP inhibitors to treat mustard induced injuries, on the one hand, or to support mustard based chemotherapy, on the other hand.

4.1. (i) CEES and HN2 as SM surrogates

SM is classified as a Schedule 1 chemical warfare agent and its use is regulated by the Chemical Weapons Convention. Research on SM related molecular mechanisms is of high interest for several reasons ([Mangerich and Esser, 2014](#)): (i) mustard exposure is still a considerable threat, as this may occur during the destruction of existing SM stockpiles or SM release by terroristic attacks or uncontrolled military actions. In order to develop efficient medical countermeasures for mustard induced pathologies, a detailed understanding of the underlying toxicological mechanisms is essential. (ii) SM derivatives, i.e., several nitrogen mustards, are being used as chemotherapeutics for the treatment of various types of cancers. To this end, an understanding of the molecular mechanisms of mustard compounds may help render such therapies more efficient and tolerable. For legal reasons, research partners (e.g., universities or commercial enterprises) of the above mentioned expert laboratories cannot conduct experiments with SM and thus, there is a significant need to define the most appropriate surrogate substances for SM for their research. The mono functional alkylating agent CEES is widely used as such a substitute, because it represents the mono functional analogue to SM [[Fig. 1](#), ([Inturi et al., 2011](#); [Jain et al., 2011](#); [Jowsey et al., 2009](#);

[Sayer et al., 2010](#); [Wang et al., 2012](#)]]. In addition, the bi functional nitrogen mustard is used as a surrogate agent, because it takes into account the bi functional reaction mechanism of SM [[Fig. 1](#), ([Jain et al., 2013](#); [Tewari Singh et al., 2013](#); [Wang et al., 2012](#)]]. However, which of the two substances represents the more appropriate SM substitute is a matter of debate. Our data suggest that HN2 is actually the preferable substitute to study SM related molecular mechanisms. Like SM, it is a bifunctional agent and its simple structure without any voluminous substituents facilitates rapid reaction with biological macromolecules and thus, high cytotoxicity. In contrast, chlorambucil is still a bifunctional agent, but a large substituent has been introduced to limit its toxicity and as shown in [Fig. 1](#), an approx. 4 fold concentration (compared to SM or HN2) is required to induce a similar 50% cytotoxicity. CEES is a monofunctional alkylating agent and its toxicity profile in HaCaT cells is very different from all other compounds tested: concentrations of up to 333 μ M were tolerated without any apparent effect on cell vitality, even 1000 μ M only induced a minor, 10% decline, whereas 3000 μ M CEES resulted in almost 100% cell death under the conditions used in experiments shown in [Fig. 1](#). In summary, our results show that with some limitations HN2, a bifunctional alkylating agent with a simple structure closely resembling SM, represents an appropriate surrogate to study SM related toxicity.

Similar results were achieved in clonogenic survival ([Fig. 6](#)) cytotoxicity profiles, in which SM and HN2 exhibit similar LD50 values in HaCaT cells, which are >10 fold lower than that of CEES. Furthermore, the PARYlation responses induced by SM and HN2 are much more similar than the one induced by CEES. This holds true, both for quantitative aspects of PAR formation ([Fig. 2D](#)) as well as for time dependent PARYlation dynamics ([Fig. 3](#)). This may be attributed to the fact that SM and HN2 induce DNA crosslinks, whereas CEES does not form this kind of damage. These *in vitro* results are consistent with the tissue pathology induced by mustard compounds. Thus, while SM and HN2 display similar pathological potencies in mice, CEES needs to be applied in $\sim$ 100 fold higher concentrations to observe comparable effects ([Tewari Singh et al., 2013](#); [Wang et al., 2012](#)). Furthermore, the same holds true for the chemical reactivity of these substances ([Wang et al., 2012](#)). Differences between SM and CEES induced toxicity also became apparent, when comparing apoptosis and necrosis

Fig. 8. Analysis of micronuclei frequencies in HaCaT cells upon mustard treatment $\pm$ PARP inhibition. A. Representative image of micronucleus formation in binucleated HaCaT cells. Cells were incubated for 24 h with cytochalasin-B, fixed, stained with an anti-tubulin antibody and Hoechst33342, and analyzed by laser scanning microscopy. White arrow indicates micronucleus. Scale bar indicates 10 μ m. B-D. Cells were pre-incubated $\pm$ ABT888 (1 μ M) for 30 min, then treated with SM (B), CEES (C), HN2 (D) at concentrations as indicated for 1 h, incubated $\pm$ ABT888 (1 μ M) and cytochalasin-B for another 24 h, and subjected to micronucleus analysis under blinded condition. PBS refers to PBS control, EtOH to ethanol solvent control. Data represent means $\pm$ SD from 3 independent experiments. Statistical evaluation was performed using two-way ANOVA testing followed by Sidak's multiple comparison testing. * $P < 0.05$.

induction by these compounds in the presence of PARP inhibitors. While PARP inhibition induced a shift from necrosis to apoptosis in SM treated cells (Kehe et al., 2008), our current analysis did not reveal such a shift for CEES treated cells. Furthermore, a proteomics approach studying membrane associated target proteins of SM and CEES identified differential subsets of proteins that were modified by SM and CEES, underscoring the different cellular mechanisms by which these two compounds exert their toxic effects (Sayer et al., 2010). Taken together, this implies that caution is indicated when using CEES as the only surrogate to study SM related molecular mechanisms, since CEES and SM may trigger considerably different molecular responses in the cell. Although in many aspects HN2 represents a more suitable SM substitute, also in this case, results need to be interpreted with caution, since even SM and HN2 display substance specific effects. This is exemplified by the finding that ABT888 treatment caused a stronger sensitization of HaCaT cells with regards to clonogenic survival, when treated with SM as compared to HN2 treatment (Fig. 6).

In conclusion, we recommend to study CEES and HN2 side by side when using these substances as SM substitutes to analyze mustard induced molecular toxicity, because based on their structure and reactivities, both compounds take into account complementary aspects of SM related chemistry and toxicology.

4.2. (ii) PARylation actively participates in the mustard induced cellular stress response:

Although a considerably body of evidence has been collected showing that mustards induce PARP activation, these reports were rather incidental and evidence for functional consequences of this

response were inconsistent. The present systematic study reveals substance specific PARylation responses in HaCaT cells after treatment with either mono or bi functional sulfur and nitrogen mustards and demonstrates that PARylation plays a significant role in cellular stress response with functional consequences on mustard induced cytotoxicity and genotoxicity. It is important to note that it is not self evident that mustards induce PARylation, since these substances mainly induce bulky base adducts and DNA crosslinks, but usually no or only a minor fraction of bona fide base excision repair (BER) substrates or direct DNA strand breaks. PARP1, however, which is the main contributor to CEES induced PARylation (Debiak et al., 2015), is considered to be mainly involved in BER and single strand break repair (Robert et al., 2013). On the other hand, there is increasing evidence that PARylation also participates in nucleotide excision repair (NER) (Fischer et al., 2014), non homologous end joining (NHEJ), and homologous recombination (HR), the latter one playing an important role during inter strand crosslink repair (ICL) repair (Pines et al., 2013). HR was reported to be the major repair pathway protecting cells against acute SM and HN2 toxicity, with NER and NHEJ also contributing to cell survival (Inturi et al., 2014; Jowsey et al., 2010, 2012; Moller et al., 2000; Muller et al., 2000). In contrast, repair of CEES induced DNA monoadducts seem to rely mostly on BER and NER pathways (Jowsey et al., 2009; Matijasevic et al., 2001). Thus, even though mustards do not induce DNA strand breaks directly, strand breaks can occur in response to mustard treatment by enzymatic processes that actively introduce them in the course of DNA repair processes. In line with this view, several studies revealed the induction of DNA strand break markers, such as phosphorylation of H2A.X, ATM/ATR, CHK1/2 and p53, upon

mustard treatment (Debiak et al., 2011; Inturi et al., 2011; Jain et al., 2011; Jowsey et al., 2009, 2012) (Fig. S3). Reconciling these findings, it is likely that the observed substance specific PARylation dynamics represent the occurrence of DNA strand breaks, introduced by endonucleases in the course of the different DNA repair mechanisms in action (Fig. 9). Interestingly, a biphasic PARylation response, as observed in the present study after treatment with bi functional mustards, resembles very much the cellular response after UVB irradiation that also resulted in initial PARP activation after 5 min and then later between 60 and 120 min (Vodenicharov et al., 2005), suggesting that BER and NER related mechanisms are at work here.

In conclusion, these results suggest a participation of PARP in the mustard induced cellular stress responses to repair different kinds of DNA lesions in order to maintain cellular homeostasis after mustard treatment. Consistent with this, studies by Bhat et al. suggested a role of PARylation in mustard induced DNA repair by stabilizing DNA ligase I, consistent with a decreased half life of ligase I and reduced DNA repair in PARP deficient keratinocytes (Bhat et al., 2006, 2000). Future studies are required to define the exact molecular basis for PARP activation during mustard induced genotoxic stress response and to unravel the down stream signaling effects of mustard induced PARylation.

4.3. PARP inhibitors should be considered with caution as a treatment option of SM induced pathologies, but may represent a possible chemosensitizer for mustard based cancer therapies

Animal experiments showed that PARP inhibition and/or boosting of NAD⁺ levels can reduce SM induced pathology, suggesting that PARP inhibitors may be used as a medical countermeasure for SM related injuries (Cowan et al., 2003; Gross et al., 1985; Mol et al., 1991; Yourick et al., 1991, 1993; Zhang et al.,

1995). Regarding the mechanism of action of such a treatment, it has been suggested, that PARP inhibition induces a switch in the mode of cell death by inhibiting necrosis but driving cells into apoptosis, which, in combination with inhibition of NF κ B related gene transcription, counteracts SM induced inflammatory responses (Bai and Virag, 2012a; Kehe et al., 2008; Meier and Millard, 1998; Rosenthal et al., 2001). However, an important caveat in this rationale is given by the double edged role of PARylation in mustard induced stress response, because in addition to its pro inflammatory role, PARPs are known to function as caretakers of genomic stability. Therefore, one of the key questions of the present study was to test if PARP inhibition in combination with mustard treatment may lead to potentiation of mustard induced genotoxicity, thereby potentially increasing the risk for cell transformation and carcinogenesis in the long run (Fig. 9). As is evident from our functional data, PARP inhibition can sensitize cells to various endpoints of mustard induced toxicity (Figs. 5, 6 and 8) including micronucleus formation. Although the potentiating effects of PARP inhibition were moderate to mild, it has to be stressed that, e.g., micronucleus formation represents a rather drastic form of genomic damage. Therefore, it is expected that more subtle forms of genomic alterations, such as mutations, microdeletions, and stalled replication forks, precede micronucleus formation. Considering these results, we advise caution regarding the potential use of PARP inhibitors to treat non lethal mustard related pathologies, since at present it cannot be excluded that PARP inhibition leads to a potentiation of SM induced carcinogenesis in the long run. Nevertheless, pharmacological PARP inhibition may be considered as a treatment option for severe cases, i.e., for patients that were exposed to lethal doses of SM. In this case, the development of PARP inhibitors as SM antidotes may represent a potential strategy to pursue, since several PARP inhibitors are close to approval for their usage in

Fig. 9. Scheme summarizing potential mechanisms of mustard-induced genotoxicity, PARP activation and consequences of its inhibition. This scheme is based on findings of this study and evidence from the literature as discussed in the text body. Mustard agents induce DNA adducts. These adducts exert direct and indirect cytotoxic effects by either triggering genetic instability/mutagenesis or driving cells into death via apoptosis/necrosis. In the course of the repair of these DNA adducts, DNA strand breaks can occur that may lead to PARP1 binding and its activation, thereby supporting subsequent DNA repair steps. PARP1 can contribute to tissue inflammation via PARP1-dependent cell death or transcriptional activation of NF- κ B. The effect of pharmacological PARP inhibition is two-fold: On the one hand, as shown previously, PARP inhibition reduces mustard-induced tissue inflammation, however, on the other hand, PARP inhibition can potentiate mustard-induced genotoxicity (this study), thereby potentially driving carcinogenesis in the long run.

cancer therapies, which paves the way for potential off label usage. Furthermore, since PARP inhibitors potentiated mustard induced toxicity, it is tempting to speculate that their application may be useful as chemosensitizers of mustard base cancer therapies. Interestingly, some studies indeed support such a scenario. For example PARP inhibitors potentiated the effect of cyclophosphamide in preclinical tumor models (Donawho et al., 2007; Norris et al., 2014), and clinical trials have been set up to further examine the tolerability and effectiveness of this drug combination (Kummar et al., 2012) (www.clinicaltrials.gov). Our results with chlorambucil and HN2 suggest that it may be worthwhile to consider also other combinations of PARP inhibitors with mustard based chemotherapeutics as treatment options.

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References

- Bai, P., Virag, L., 2012a. Role of poly(ADP-ribose) polymerases in the regulation of inflammatory processes. *FEBS Lett.* 586, 3771–3777.
- Bai, P., Virag, L., 2012b. Role of poly(ADP-ribose) polymerases in the regulation of inflammatory processes. *FEBS Lett.*
- Barkauskaite, E., Jankevicius, G., Ladurner, A.G., Ahel, I., Timinszky, G., 2013. The recognition and removal of cellular poly(ADP-ribose) signals. *FEBS J.*
- Batal, M., Boudry, I., Mouret, S., Wartelle, J., Emorine, S., Bertoni, M., Berard, I., Clery-Barraud, C., Douki, T., 2013. Temporal and spatial features of the formation of DNA adducts in sulfur mustard-exposed skin. *Toxicol. Appl. Pharmacol.* 273, 644–650.
- Batal, M., Boudry, I., Mouret, S., Clery-Barraud, C., Wartelle, J., Berard, I., Douki, T., 2014. DNA damage in internal organs after cutaneous exposure to sulphur mustard. *Toxicol. Appl. Pharmacol.*
- Beneke, S., 2012. Regulation of chromatin structure by poly(ADP-ribose) ation. *Front. Genet.* 3, 169.
- Bennett, R.A., Behrens, E., Zinn, A., Duncheon, C., Lamkin, T.J., 2014. Mustard gas surrogate, 2-chloroethyl ethylsulfide (2-CEES), induces centrosome amplification and aneuploidy in human and mouse cells: 2-CEES induces centrosome amplification and chromosome instability. *Cell Biol. Toxicol.* 30, 195–205.
- Bhat, K.R., Benton, B.J., Rosenthal, D.S., Smulson, M.E., Ray, R., 2000. Role of poly (ADP-ribose) polymerase (PARP) in DNA repair in sulfur mustard-exposed normal human epidermal keratinocytes (NHEK). *J. Appl. Toxicol.* 20 (Suppl. 1), S13–17.
- Bhat, K.R., Benton, B.J., Ray, R., 2006. Poly (ADP-ribose) polymerase (PARP) is essential for sulfur mustard-induced DNA damage repair, but has no role in DNA ligase activation. *J. Appl. Toxicol.* 26, 452–457.
- Boukamp, P., Petrussevska, R.T., Breitkreutz, D., Hornung, J., Markham, A., Fusenig, N. E., 1988. Normal keratinization in a spontaneously immortalized aneuploid human keratinocyte cell line. *J. Cell Biol.* 106, 761–771.
- Boulware, S., Fields, T., McIvor, E., Powell, K.L., Abel, E.L., Vasquez, K.M., MacLeod, M. C., 2012. 2,6-Dithiopurine, a nucleophilic scavenger, protects against mutagenesis in mouse skin treated in vivo with 2-(chloroethyl) ethyl sulfide, a mustard gas analog. *Toxicol. Appl. Pharmacol.* 263, 203–209.
- Cowan, F.M., Broomfield, C.A., Lenz, D.E., Smith, W.J., 2003. Putative role of proteolysis and inflammatory response in the toxicity of nerve and blister chemical warfare agents: implications for multi-threat medical countermeasures. *J. Appl. Toxicol.* 23, 177–186.
- Curtin, N.J., Szabo, C., 2013. Therapeutic applications of PARP inhibitors: anti therapy and beyond. *Mol. Aspects Med.*
- Debiak, M., Kehe, K., Bürkle, A., 2009. Role of poly(ADP-ribose) polymerase in sulfur mustard toxicity. *Toxicology* 263, 20–25.
- Debiak, M., Panas, A., Steinritz, D., Kehe, K., Bürkle, A., 2011. High-throughput analysis of DNA interstrand crosslinks in human peripheral blood mononuclear cells by automated reverse FADU assay. *Toxicology* 280, 53–60.
- Debiak, M., Lex, K., Ponath, V., Burckhardt-Boer, W., Thiermann, H., Steinritz, D., Schmidt, A., Mangerich, A., Bürkle, A., 2015. Immunohistochemical analysis of poly (ADP-ribose)ylation in HaCaT keratinocytes induced by the mono-alkylating agent 2-chloroethyl ethyl sulfide (CEES): impact of experimental conditions. *Toxicol Lett.* 244, 72–80.
- Donawho, C.K., Luo, Y., Luo, Y., Penning, T.D., Bauch, J.L., Bouska, J.J., Bontcheva-Diaz, V.D., Cox, B.F., DeWeese, T.L., Dillehay, L.E., Ferguson, D.C., Ghoreishi-Haack, N.S., Grimm, D.R., Guan, R., Han, E.K., Holley-Shanks, R.R., Hristov, B., Idler, K.B., Jarvis, K., Johnson, E.F., Kleinberg, L.R., Klinghofer, V., Lasko, L.M., Liu, X., Marsh, K.C., McGonigal, T.P., Meulbroek, J.A., Olson, A.M., Palma, J.P., Rodriguez, L.E., Shi, Y., Stavropoulos, J.A., A.C., Zhu, G.D., Rosenberg, S.H., Giranda, V.L., Frost, D.J., 2007. ABT-888, an orally active poly(ADP-ribose) polymerase inhibitor that potentiates DNA-damaging agents in preclinical tumor models. *Clin. Cancer Res.* 13, 2728–2737.
- Fenech, M., 2007. Cytokinesis-block micronucleus cytome assay. *Nat Protoc.* 2, 1084–1104.
- Fidder, A., Moes, G.W., Scheffer, A.G., van der Schans, G.P., Baan, R.A., de Jong, L.P., Benschop, H.P., 1994. Synthesis, characterization, and quantitation of the major adducts formed between sulfur mustard and DNA of calf thymus and human blood. *Chem. Res. Toxicol.* 7, 199–204.
- Fischer, J.M., Popp, O., Gebhard, D., Veith, S., Fischbach, A., Beneke, S., Leitenstorfer, A., Bergemann, J., Scheffner, M., Ferrando-May, E., Mangerich, A., Bürkle, A., 2014. Poly(ADP-ribose)-mediated interplay of XPA and PARP1 leads to reciprocal regulation of protein function. *FEBS J.* 281, 3625–3641.
- Franken, N.A., Rodermond, H.M., Stap, J., Haveman, J., van Bree, C., 2006. Clonogenic assay of cells in vitro. *Nat. Protoc.* 1, 2315–2319.
- Gibson, B.A., Kraus, W.L., 2012. New insights into the molecular and cellular functions of poly(ADP-ribose) and PARPs. *Nat. Rev. Mol. Cell Biol.* 13, 411–424.
- Gilman, A., Philips, F.S., 1946. The biological actions and therapeutic applications of the B-chloroethyl amines and sulfides. *Science* 103, 409–436.
- Gross, C.L., Meier, H.L., Papirmeister, B., Brinkley, F.B., Johnson, J.B., 1985. Sulfur mustard lowers nicotinamide adenine dinucleotide concentrations in human skin grafted to athymic nude mice. *Toxicol. Appl. Pharmacol.* 81, 85–90.
- Hinshaw, D.B., Lodhi, I.J., Hurley, L.L., Atkins, K.B., Dabrowska, M.L., 1999. Activation of poly [ADP-Ribose] polymerase in endothelial cells and keratinocytes: role in an in vitro model of sulfur mustard-mediated vesication. *Toxicol. Appl. Pharmacol.* 156, 17–29.
- Hottiger, M.O., 2015. Nuclear ADP-ribosylation and its role in chromatin plasticity, differentiation, and epigenetics. *Annu. Rev. Biochem.*
- Inturi, S., Tewari-Singh, N., Gu, M., Shrotriya, S., Gomez, J., Agarwal, C., White, C.W., Agarwal, R., 2011. Mechanisms of sulfur mustard analog 2-chloroethyl ethyl sulfide-induced DNA damage in skin epidermal cells and fibroblasts. *Free Radic. Biol. Med.* 51, 2272–2280.
- Inturi, S., Tewari-Singh, N., Agarwal, C., White, C.W., Agarwal, R., 2014. Activation of DNA damage repair pathways in response to nitrogen mustard-induced DNA damage and toxicity in skin keratinocytes. *Mutat. Res.*
- Jacobson, E.L., Jacobson, M.K., 1976. Pyridine nucleotide levels as a function of growth in normal and transformed 3T3 cells. *Arch. Biochem. Biophys.* 175, 627–634.
- Jain, A.K., Tewari-Singh, N., Gu, M., Inturi, S., White, C.W., Agarwal, R., 2011. Sulfur mustard analog, 2-chloroethyl ethyl sulfide-induced skin injury involves DNA damage and induction of inflammatory mediators, in part via oxidative stress, in SKH-1 hairless mouse skin. *Toxicol. Lett.* 205, 293–301.
- Jain, A.K., Tewari-Singh, N., Inturi, S., Orlicky, D.J., White, C.W., Agarwal, R., 2013. Histopathological and immunohistochemical evaluation of nitrogen mustard-induced cutaneous effects in SKH-1 hairless and C57BL/6 mice. *Exp. Toxicol. Pathol.*
- Jowsey, P.A., Williams, F.M., Blain, P.G., 2009. DNA damage, signalling and repair after exposure of cells to the sulphur mustard analogue 2-chloroethyl ethyl sulphide. *Toxicology* 257, 105–112.
- Jowsey, P.A., Williams, F.M., Blain, P.G., 2010. The role of homologous recombination in the cellular response to sulphur mustard. *Toxicol. Lett.* 197, 12–18.
- Jowsey, P.A., Williams, F.M., Blain, P.G., 2012. DNA damage responses in cells exposed to sulphur mustard. *Toxicol. Lett.* 209 (1), 1–10.
- Kehe, K., Raithel, K., Kreppel, H., Jochum, M., Worek, F., Thiermann, H., 2008. Inhibition of poly(ADP-ribose) polymerase (PARP) influences the mode of sulfur mustard (SM)-induced cell death in HaCaT cells. *Arch. Toxicol.* 82, 461–470.
- Kehe, K., Balszuweit, F., Steinritz, D., Thiermann, H., 2009. Molecular toxicology of sulfur mustard-induced cutaneous inflammation and blistering. *Toxicology* 263, 12–19.
- Kehe, K., Schrettel, V., Thiermann, H., Steinritz, D., 2013. Modified immunoslot blot assay to detect hemi and sulfur mustard DNA adducts. *Chem. Biol. Interact.*
- Kraus, W.L., Hottiger, M.O., 2013. PARP-1 and gene regulation: progress and puzzles. *Mol. Aspects Med.* 34, 1109–1123.
- Krietsch, J., Rouleau, M., Pic, E., Ethier, C., Dawson, T.M., Dawson, V.L., Masson, J.Y., Poirier, G.G., Gagne, J.P., 2013. Reprogramming cellular events by poly(ADP-ribose)-binding proteins. *Mol. Aspects Med.* 34, 1066–1087.

- Kummar, S., Ji, J., Morgan, R., Lenz, H.J., Puhalla, S.L., Belani, C.P., Gandara, D.R., Allen, D., Kiesel, B., Beumer, J.H., Newman, E.M., Rubinstein, L., Chen, A., Zhang, Y., Wang, L., Kinders, R.J., Parchment, R.E., Tomaszewski, J.E., Doroshow, J.H., 2012. A phase I study of veliparib in combination with metronomic cyclophosphamide in adults with refractory solid tumors and lymphomas. *Clin. Cancer Res.* 18, 1726–1734.
- Lin, P., Bernstein, I.A., Vaughan, F.L., 1994. Failure to observe a relationship between bis-(beta-chloroethyl) sulfide-induced NAD depletion and cytotoxicity in the rat keratinocyte culture. *J. Toxicol. Environ. Health* 42, 393–405.
- Mangerich, A., Bürkle, A., 2011. How to kill tumor cells with inhibitors of poly(ADP-ribose) ation. *Int. J. Cancer* 128, 251–265.
- Mangerich, A., Bürkle, A., 2012. Pleiotropic cellular functions of PARP in longevity and aging: genome maintenance meets inflammation. *Oxid. Med. Cell Longev.* 2012, 321653.
- Mangerich, A., Esser, C., 2014. Chemical warfare in the First World War: reflections 100 years later. *Arch. Toxicol.* 88, 1909–1911.
- Martello, R., Mangerich, A., Sass, S., Dedon, P.C., Bürkle, A., 2013. Quantification of ular poly(ADP-ribose) ation by stable isotope dilution mass spectrometry reveals tissue- and drug-dependent stress response dynamics. *ACS Chem. Biol.*
- Matijasevic, Z., Volkert, M.R., 2007. Base excision repair sensitizes cells to sulfur mustard and chloroethyl ethyl sulfide. *DNA Repair (Amst)* 6, 733–741.
- Matijasevic, Z., Precopio, M.L., Snyder, J.E., Ludlum, D.B., 2001. Repair of sulfur mustard-induced DNA damage in mammalian cells measured by a host cell reactivation assay. *Carcinogenesis* 22, 661–664.
- Meier, H.L., Johnson, J.B., 1992. The determination and prevention of cytotoxic effects induced in human lymphocytes by the alkylating agent 2,2'-dichlorodiethyl sulfide (sulfur mustard, HD). *Toxicol. Appl. Pharmacol.* 113, 234–239.
- Meier, H.L., Millard, C.B., 1998. Alterations in human lymphocyte DNA caused by sulfur mustard can be mitigated by selective inhibitors of poly(ADP-ribose) polymerase. *Biochim. Biophys. Acta* 1404, 367–376.
- Meier, H.L., Gross, C.L., Papirmeister, B., 1987. 2,2'-Dichlorodiethyl sulfide (sulfur mustard) decreases NAD⁺ levels in human leukocytes. *Toxicol. Lett.* 39, 109–122.
- Meier, H.L., Millard, C., Moser, J., 2000. Poly(ADP-ribose) polymerase inhibitors regulate the mechanism of sulfur mustard-initiated cell death in human lymphocytes. *J. Appl. Toxicol.* 20 (Suppl. 1), S93–100.
- Meier, H.L., 1996. The time-dependent effect of 2,2'-dichlorodiethyl sulfide (sulfur mustard, HD, 1,1'-thiobis [2-chloroethane]) on the lymphocyte viability and the kinetics of protection by poly(ADP-ribose) polymerase inhibitors. *Cell Biol. Toxicol.* 12, 147–153.
- Mol, M.A., van de Ruit, A.M., Kluivers, A.W., 1989. NAD⁺ levels and glucose uptake of cultured human epidermal cells exposed to sulfur mustard. *Toxicol. Appl. Pharmacol.* 98, 159–165.
- Mol, M.E., de Vries, R., Kluivers, A.W., 1991. Effects of nicotinamide on biochemical changes and microblistering induced by sulfur mustard in human skin organ cultures. *Toxicol. Appl. Pharmacol.* 107, 439–449.
- Moller, P., Wassermann, K., Damgaard, J., Nexø, B.A., Wallin, H., 2000. Sensitivity to nitrogen mustard relates to the ability of processing DNA damage in Chinese hamster ovary cells. *Pharmacol. Toxicol.* 86, 169–177.
- Muller, C., Calsou, P., Salles, B., 2000. The activity of the DNA-dependent protein kinase (DNA-PK) complex is determinant in the cellular response to nitrogen mustards. *Biochimie* 82, 25–28.
- Neidle, S., Thurston, D.E., 2005. Chemical approaches to the discovery and development of cancer therapies. *Nat. Rev. Cancer* 5, 285–296.
- Norris, R.E., Adamson, P.C., Nguyen, V.T., Fox, E., 2014. Preclinical evaluation of the PARP inhibitor, olaparib, in combination with cytotoxic chemotherapy in pediatric solid tumors. *Pediatr. Blood Cancer* 61, 145–150.
- Papirmeister, B., Gross, C.L., Meier, H.L., Petrali, J.P., Johnson, J.B., 1985. Molecular basis for mustard-induced vesication. *Fundam. Appl. Toxicol.* 5, S134–149.
- Paromov, V., Brannon, M., Kumari, S., Samala, M., Qui, M., Smith, M., Stone, W.L., 2011a. Sodium pyruvate modulates cell death pathways in HaCaT keratinocytes exposed to half-mustard gas. *Int. J. Toxicol.* 30, 197–206.
- Paromov, V., Kumari, S., Brannon, M., Kanaparthi, N.S., Yang, H., Smith, M.G., Stone, W.L., 2011b. Protective effect of liposome-encapsulated glutathione in a human epidermal model exposed to a mustard gas analog. *J. Toxicol.* 2011, 109516.
- Pines, A., Mullenders, L.H., van Attikum, H., Luijsterburg, M.S., 2013. Touching base with PARPs: moonlighting in the repair of UV lesions and double-strand breaks. *Trends Biochem. Sci.* 38, 321–330.
- Powell, K.L., Boulware, S., Thames, H., Vasquez, K.M., MacLeod, M.C., 2010. 2,6-Dithiopurine blocks toxicity and mutagenesis in human skin cells exposed to sulfur mustard analogues, 2-chloroethyl ethyl sulfide and 2-chloroethyl methyl sulfide. *Chem. Res. Toxicol.* 23, 497–503.
- Robert, I., Karicheva, O., Reina San Martín, B., Schreiber, V., Dantzer, F., 2013. Functional aspects of PARylation in induced and programmed DNA repair processes: preserving genome integrity and modulating physiological events. *Mol. Aspects Med.* 34, 1138–1152.
- Rosenthal, D.S., Simbulan-Rosenthal, C.M., Iyer, S., Spoonde, A., Smith, W., Ray, R., Smulson, M.E., 1998. Sulfur mustard induces markers of terminal differentiation and apoptosis in keratinocytes via a Ca²⁺-calmodulin and caspase-dependent pathway. *J. Invest. Dermatol.* 111, 64–71.
- Rosenthal, D.S., Simbulan-Rosenthal, C.M., Liu, W.F., Velená, A., Anderson, D., Benton, B., Wang, Z.Q., Smith, W., Ray, R., Smulson, M.E., 2001. PARP determines the mode of cell death in skin fibroblasts, but not keratinocytes, exposed to sulfur mustard. *J. Invest. Dermatol.* 117, 1566–1573.
- Sayer, N.M., Whiting, R., Green, A.C., Anderson, K., Jenner, J., Lindsay, C.D., 2010. Direct binding of sulfur mustard and chloroethyl ethyl sulphide to human cell membrane-associated proteins; implications for sulfur mustard pathology. *J. Chromatogr. B Anal. Technol. Biomed. Life Sci.* 878, 1426–1432.
- Seker, H., Bertram, B., Bürkle, A., Kaina, B., Pohl, J., Koepsell, H., Wiesser, M., 2000. Mechanistic aspects of the cytotoxic activity of glufosfamide, a new tumour therapeutic agent. *Br. J. Cancer* 82, 629–634.
- Shakarjian, M.P., Heck, D.E., Gray, J.P., Sinko, P.J., Gordon, M.K., Casillas, R.P., Heindel, N.D., Gerecke, D.R., Laskin, D.L., Laskin, J.D., 2010. Mechanisms mediating the vesicant actions of sulfur mustard after cutaneous exposure. *Toxicol. Sci.* 114, 5–19.
- Smith, W.J., Gross, C.L., Chan, P., Meier, H.L., 1990. The use of human epidermal keratinocytes in culture as a model for studying the biochemical mechanisms of sulfur mustard toxicity. *Cell Biol. Toxicol.* 6, 285–291.
- Steinritz, D., Schmidt, A., Simons, T., Ibrahim, M., Morguet, C., Balszuweit, F., Thiermann, H., Kehe, K., Bloch, W., Bolck, B., 2014. Chlorambucil (nitrogen mustard) induced impairment of early vascular endothelial cell migration—effects of alpha-linolenic acid and N-acetylcysteine. *Chem. Biol. Interact.* 219c, 143–150.
- Tewari-Singh, N., Gu, M., Agarwal, C., White, C.W., Agarwal, R., 2010. Biological and molecular mechanisms of sulfur mustard analogue-induced toxicity in JB6 and HaCaT cells: possible role of ataxia telangiectasia-mutated/ataxia telangiectasia-Rad3-related cell cycle checkpoint pathway. *Chem. Res. Toxicol.* 23, 1034–1044.
- Tewari-Singh, N., Jain, A.K., Inturi, S., White, C.W., Agarwal, R., 2013. Clinically-relevant cutaneous lesions by nitrogen mustard: useful biomarkers of vesicants skin injury in SKH-1 hairless and C57BL/6 mice. *PLoS One* 8, e67557.
- Tewari-Singh, N., Inturi, S., Jain, A.K., Agarwal, C., Orlicky, D.J., White, C.W., Agarwal, R., Day, B.J., 2014a. Catalytic antioxidant AEOL 10150 treatment ameliorates sulfur mustard analog 2-chloroethyl ethyl sulfide associated cutaneous toxic effects. *Free Radic. Biol. Med.*
- Tewari-Singh, N., Jain, A.K., Orlicky, D.J., White, C.W., Agarwal, R., 2014b. Cutaneous injury-related structural changes and their progression following topical nitrogen mustard exposure in hairless and haired mice. *PLoS ONE* 9, e85402.
- Vodenicharov, M.D., Ghodgaonkar, M.M., Halappanavar, S.S., Shah, R.G., Shah, G.M., 2005. Mechanism of early biphasic activation of poly(ADP-ribose) polymerase-1 in response to ultraviolet B radiation. *J. Cell Sci.* 118, 589–599.
- Wang, Q.Q., Begum, R.A., Day, V.W., Bowman-James, K., 2012. Sulfur, oxygen, and nitrogen mustards: stability and reactivity. *Org. Biomol. Chem.* 10, 8786–8793.
- Yourick, J.J., Clark, C.R., Mitcheltree, L.W., 1991. Niacinamide pretreatment reduces microvesicle formation in hairless guinea pigs cutaneously exposed to sulfur mustard. *Fundam. Appl. Toxicol.* 17, 533–542.
- Yourick, J.J., Dawson, J.S., Benton, C.D., Craig, M.E., Mitcheltree, L.W., 1993. Pathogenesis of 2,2'-dichlorodiethyl sulfide in hairless guinea pigs. *Toxicology* 84, 185–197.
- Yue, L., Wei, Y., Chen, J., Shi, H., Liu, Q., Zhang, Y., He, J., Guo, L., Zhang, T., Xie, J., Peng, S., 2014. The abundance of four sulfur mustard-dna adducts ex vivo and in vivo revealed by simultaneous quantification in stable isotope dilution-ultrahigh performance liquid chromatography–tandem mass spectrometry. *Chem. Res. Toxicol.*
- Zhang, Z., Riviere, J.E., Monteiro-Riviere, N.A., 1995. Evaluation of protective effects of sodium thiosulfate, cysteine, niacinamide and indomethacin on sulfur mustard-treated isolated perfused porcine skin. *Chem.-Biol. Interact.* 96, 249–262.