Bundeswehr Institute of Pharmacology and Toxicology

16th Medical Chemical Defense Conference

100 years after the first use of sulfur mustard - re-emerging threats of chemical warfare agents and current state of medical research

April 05 - 06, 2017, Munich

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16th Medical Chemical Defense Conference

Dear congress participants, dear readers of the Wehrmedizinische Monatsschrift,

when we started to prepare the 16th Medical Chemical Defense Conference which was held from April 05 - 06, 2017 in Munich, we did not have a presentiment that this cruel nerve gas attack against the civilian population would happen in Syria just one day prior to our meeting. This incidence demonstrated again that – despite of international agreements and established control mechanisms – chemical weapons are still available for armed forces, terrorists and even criminals and that they are willing to use them in a contemptuous manner. This underlines the importance of international cooperation among physicians, biologists, chemists, pharmacists, veterinarians and also engineers to develop analytics, drugs, technology and procedures in order to protect our troops and the civilian population against chemical weapons. An international conference like our meeting in Munich will help to pave the way towards better protection and treatment, which is unfortunately only available for some chemical warfare agents.

We selected a painting from John Singer Sargent with the title "Gassed" as cover picture of this year's conference booklet and this supplement. The painting depicts the aftermath of a mustard gas attack during World War I, with a line of wounded soldiers walking towards a dressing station. Sargent was commissioned by the British War Memorials Committee to document the war and visited the Western Front in July 1918 spending time with the Guards Division near Arras, and then with the American Expeditionary Forces near Ypres. The painting was finished in March 1919 and voted picture of the year by the Royal Academy of Arts in 1919. It is now held by the Imperial War Museum in London. There could not be a better painting to introduce our this year's conference that has the topic:

100 years after the first use of sulfur mustard re-emerging threats of chemical warfare agents and current state of medical research

The first deployment of sulfur mustard took place on July 12, 1917 in Ypres, Belgium, almost exactly 100 years ago. More than 50,000 tons of chemical warfare agents (CWA) including chlorine, phosgene, and mustard gas were deployed by both sides during World War I. Official figures estimate about 1.3 million casualties directly caused by chemical warfare agents during the course of the war. Based on that, chemical warfare was abandoned by the Geneva Protocol in 1925 and later on by the Chemical Weapon Convention (CWC), with 192 member states as of April 2017. However, as we have all recognized, times have newly changed.

The use of nerve and blister agents in Syria, as mentioned above, and Iraq demonstrates that chemical warfare has changed its face: from large-scale battlefield deployment towards malicious terror or asymmetric incidents. The threat arising from CWA is as great as never before. Although some well-established therapies against CWA poisoning do exist, lacking antidotes or specific therapeutics for sulfur mustard injuries and also some nerve agents, e.g. soman, present us with major medical challenges. These facts underline the ultimate need of cutting-edge scientific research in order to face the reappearing threats of chemical warfare agents.

More than 200 experts from 29 different countries – Hawaii to Japan or Brazil to Norway – participated in this year´s congress. We had 27 conference lectures from renowned speakers, addressing a plethora of different topics. These included sessions with historical aspects of chemical warfare starting in World War I, the current use of CWA in Syria and Iraq, but also top-class scientific presentations reporting about up-to-date fields of basic research with special focus on the molecular toxicology of sulfur mustard, toxic lung injury induced by pulmonary acting noxious compounds, as well as analytical methods and experimental tools.

The congress management was significantly different than in the past. The newly introduced electronic registration process made registration and package booking more convenient, and detailed information regarding conference and social program was provided in advance. However, to achieve continuous improvement we thank all participants for their suggestions and criticism which will help us "to make it better". As result of the evaluation, we will extend the next meeting in 2019 to two full days to

Tabea Zubel (Konstanz, Germany)

Bernhard Stenger (Munich, Germany)

Tamara Zorbaz (Zagreb, Croatia)

(Bilder: Julia Herbert, München)

allow more time for poster presentations and in-depth discussion.

Another novelty was the introduction of a scientific award for the best poster presented by a PhD student. The response to that was overwhelming: from the more than 50 submitted poster abstracts – as much contributions as never before – 20 abstracts submitted by PhD students were considered for the poster award. An international scientific committee of 6 scientists with outstanding reputation had selected a short-list of three excellent abstracts in advance. The three candidates presented their work in a short talk. The audience evaluated the oral presentations and its score accounted for 50 % of the overall result. The other 50 % resulted from an interview of each presenter by a local international scientific committee. All nominees convinced with excellent presentations and posters, that made the decision most difficult. At the end, jury and audience agreed in their ratings and decided as follows:

1st place: Tabea Zubel (Konstanz, Germany)
Development of a mass spectrometric platform for the quantitation of mustard-induced nucleic acid damage

2nd place: Bernhard Stenger (Munich, Germany)
Effect of N-acetylcysteine and glutathione on alkylating agents-induced TRPA1-channel activation

3rd place: Tamara Zorbaz (Zagreb, Croatia)
New uncharged potent reactivators of AChE and BChE inhibited by nerve agents

I hope that the participants are persuading the conference as the same success as I do. It was a great honor for me to host the 16th Medical Chemical Defense Conference and I say a cordial "Thank you" to my staff, especially to Major Dr. Tanja Popp and Lieutenant Colonel Dr. Dirk Steinritz, for their excellent support and professional work prior, during and after our meeting.

You will find the Abstracts of the conference lectures in the print version of this issue of the Wehrmedizinische Monatsschrift. The poster abstracts will be available in the internet version (downloadable from www.wehrmed.de and www.sanitaetsdienst-bundeswehr.de).

With best wishes

Colonel Prof. Dr. HorstThiermann
Director of the Bundeswehr Institute of Pharmacology and Toxicology, Munich, Germany

Abstracts of conference lectures

Historical Background and Current Political Situation

UN investigation of sarin use in Ghouta, Syria 2013

Å. Sellström

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Chemical weapons were used in the Ghouta area of Damascus on August 21, 2013. Therefore, on-site inspections by UN were performed over a period of three days (August 26 - 29). The suburbs Moadamiyah in West Ghouta, Ein Tarma and Zamalka in East Ghouta were visited. The inspection team included two medical doctors, two nurses, six chemical experts, security personnel and drivers. The team collected primary statements from more than fifty exposed survivors including patients, health workers and first-responders. The team also recovered 34 medical samples processed for subsequent laboratory analysis. Interviewed survivors reported an attack with shelling, followed by the onset of a common range of symptoms, including shortness of breath, disorientation, rhinorrhoea, eye irritation, blurred vision, nausea, vomiting, general weakness, and eventual loss of consciousness. Those who went to assist other community members described seeing a large number of individuals lying on the ground, many of whom were deceased or unconscious. These individuals reported observing laboured breathing and excessive salivation among a large proportion of the survivors. Several of these "first responders" also became ill. Nine nurses and seven treating physicians were interviewed by the team. In the field the clinicians observed a large number of ill or deceased persons lying in the streets without external signs of injury. Most survivors were described as being unconscious, demonstrating laboured breathing. The responders attempted to assist the survivors through the provision of first aid, decontamination with water where possible, and transfer to the nearest hospital. Eight medical records from the Zamalka Hospital were reviewed for demographics, clinical presentation, and treatment. All cases were male, with an average age of 27 years. The most common symptoms and signs documented included shortness of breath / laboured breathing, blurred vision, vomiting, miosis, and headache. All patients received atropine treatment, although dosages were not consistently recorded. The other main treatments reported included hydrocortisone and oxygen. Most of the 34 blood samples recovered tested positive for Sarin exposure. The team concluded that the positive blood and urine specimens provided definitive evidence of exposure to Sarin by a large proportion of the survivors. Furthermore, this conclusion was corroborated by the result of the clinical assessments and the interviews that documented symptoms and signs that are consistent with nerve agent exposure.

History of chemical warfare agents

A. Vale

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In recognition of the focus of the Conference, this presentation will describe the introduction as chemical warfare (CW) agents of sulphur mustard, as well as chlorine and nerve agents.

Nobel laureate, Fritz Haber (1868 - 1934), proposed that chlorine should be released directly at the enemy from cylinders placed in forward trenches. At 17.00 h on 22 April 1915, 180,000 kg of chlorine were released from 5,730 cylinders along a 6 km front against French troops who broke quickly leaving an 8 - 9 km gap in the line. On the 23 April 1915 chlorine was used against Canadian troops at the start of the Second Battle of Ypres. In the latter action there were some 7,000 casualties, of whom 350 died subsequently.

Sulphur mustard was first synthesised in pure state in 1886 by Victor Meyer (1848 - 1897). Its use as a CW agent was proposed in 1916 by Wilhelm Lommel and Wilhelm Steinkopf and it was used on the night of 12 July 1917 during the artillery bombardment of the British line preceding the Third Battle of Ypres. By the end of WWI more than 12,000 tons of mustard had been produced and there were more than 400,000 casualties (124,702 were British, including 2,308 deaths [1.85 %]). OPCW has concluded that ISIS used mustard on 3 occasions in August 2015, once in Syria (Marea in Aleppo province) and twice in Iraq.

Gerhard Schrader (1903 - 1990) was placed in charge of insecticide research at IG Farbenindustrie in 1934. One of the organophosphorus compounds prepared on 23 December 1936 proved to be highly toxic (tabun) and its value as a CW agent was recognized immediately and production began; 12,000 tons had been made by 1945. Schrader discovered sarin in 1939 and Nobel laureate, Richard Kuhn, soman in 1944. The V-nerve agents followed after WWII. Nerve agents have been used most recently in Syria.

Introduction to Sulfur Mustard

Clinical picture of sulfur mustard poisoning

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Exposure to sulfur mustard (SM) causes acute, chronic and delayed health effects. Severity is dependent on dose, mode of exposure (i.e. aerosol or liquid exposure) and exposure frequency. Already a single exposure may result in acute, chronic and delayed effects. It is accepted that SM does not cause immediate or sub-acute effects, thus the exposure remains unrecognized at first. However, new *in vitro* and *in vivo* findings, showing immediate activation of ion channels and acute effects in insects, question that fact. Acute effects after SM exposure may develop within 24 h. In case of dermal exposures, erythema, hyperpigmentation, tautness and itching are common and occur first. Blisters and ulcerations may develop afterwards and remain for weeks. Longterm health effects include pigmentation disorders, scar formation, pruritus and dry skin conditions, but also neoplasms like cherry like hemangioma or telangiectasis. In general, the same kinetic holds true for other affected organs. The eyes are the most sensitive organ and conjunctivitis develops first. However, at higher SM doses, corneal damage may

occur. While most patients recover from acute eye injury, delayed development of mustard gas keratopathy is feared. SM-induced toxic lung injury is another major problem. Lung epithelium detaches, mixes with fluid either from secretory cells or from leaking endothelium, thereby obstructing the airways. Pulmonary SM-induced long-term effects have been summarized as "Mustard Lung". Lung tumor development is another SM-induced complication. Systemic effects are related to bone-marrow dysfunctions and a plethora of consequences thereof. This talk will give a short overview about the clinical picture of SM poisoning and will link the following talks to relevant clinical aspects.

Long-term health effects of sulfur mustard exposure

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Introduction: Sulfur Mustard (SM) has toxic, mutagenic, carcinogenic and teratogenic properties and despite the well-described SM-induced acute and chronic complication, the exact underlying molecular mechanisms of these events which result in organ damage even at a long interval following a single exposure are still a matter of research.

Aims of study: The aim of this study was to investigate the clinical health status of a group of SM exposed veterans with focus on their lungs, eyes and the skin lesions, to understand the chronic delayed effect of SM exposure on DNA damage and repair pathways and finally to relate the laboratory results and the clinical findings of the patients.

Method: A full clinical examination was performed by an expert medical team to evaluate health status of SM exposed and control group then a major DNA damage protein (y-H2AX) as well as four DNA repair proteins MRE11, NBS, RAD51, and XPA was evaluated in the blood samples using western blot. Comet assay was performed to investigate the DNA damage in the blood lymphocytes of the exposed and unexposed groups.

Results: The results of this study reveal that previous exposure to SM can lead to high DNA damage and impaired repair mechanism in cells of exposed individuals which is likely attributable to SM exposure as not observed in controls. Such disorders in cellular level can justify the long life health problems among individuals exposed to SM.

Thus, manipulation of DNA damage/repair pathways might be helpful in mitigating their chronic diseases as well as for protective countermeasures against exposure to SM.

Insights in sulfur mustard ocular research experience and future directions

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The eyes are one of the main affected organs during sulfur mustard (SM) exposure. While the acute phase is characterized by erosions and severe inflammation, the delayed pathology is clinically expressed by epithelial defects, chronic inflammation and neovascularization. Based on the similarities between rabbit and human corneas we used our *in vivo* vapor exposure system in rabbits to study different aspects of SM-induced ocular injury, aiming to find therapeutic measures.

Rabbit eyes were exposed to SM vapor and a clinical follow-up was carried out using the slit lamp microscope. Additional non-invasive monitoring included pachymetry, specular microscopy, impression cytology and tear fluid collection. Histology, IHC, biochemistry and molecular biology methods were applied on the ocular surface tissues.

Typical SM-induced ocular injury developed, starting with an acute phase in all of the exposed eyes and followed by a delayed pathology in 50 % - 80 % of the eyes. Severe and chronic inflammation, impaired corneal innervation, damaged corneal endothelial cell layer, decreased goblet cell density and delayed limbal stem cell deficiency were observed in the exposed eyes. Elevations in MMP-9, VEGF, IL-1α, MCP-1 and IL-8 levels were found in both tear fluid and corneal samples. Treatments with anti-inflammatory drugs, anti-VEGF compounds or doxycycline reduced the severity of the ocular injury.

Studying SM-exposed rabbit eyes shed light on the pathological mechanisms involved in the different stages of the ocular injury and pointed out towards novel therapeutic strategies that were shown to be beneficial *in vivo*. Aiming to improve the therapeutic measures and the monitoring abilities, we continue to look for additional factors that play a role in this complex ocular pathology.

Therapeutic approaches countering sulfur mustard toxicity

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Despite decades of research, the biochemical mechanisms of toxicity of sulfur mustard (SM) are not fully understood; consequently there is no fielded, evidence based treatment strategy for the management of SM toxicity. Medical management relies on prevention of exposure, decontamination and symptomatic treatment of the injury.

SM readily penetrates epithelial tissues causing damage to the eye, respiratory tract and skin. SM is a reactive, bifunctional, alkylating agent, which interacts with nucleophiles on the cell membrane, at intracellular sites, and with nucleic acids. SM alkylates DNA, RNA, proteins and phospholipids affecting a variety of cell functions. Other potential mechanisms of cell death relate to rapid inactivation of sulfhydryl containing proteins and peptides, such as glutathione (GSH); removing one of the major cellular defence mechanisms against electrophilic compounds and oxidants. The toxic effects of SM may result from direct damage induced by alkylation or by production of reactive oxygen species.

Pulmonary tract lesions are the primary source of morbidity and mortality. Treatment for the acute pulmonary injury is compounded by the difficulty of intervening early in the disease process, and the lack of specific pharmacologic therapies. Physiological consequences of SM-induced lung injury are similar to those following exposure to other toxic chemicals; the lung is

no longer able to function efficiently. This presentation will consider treatment options and future research requirements.

Toxicokinetics and verification of exposure to sulfur mustard

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An overview is presented on the state of knowledge concerning the toxicokinetics of sulfur mustard, as well as methods that can be used to assess an exposure to this agent.

The toxicokinetics of intact sulfur mustard in blood and tissues of various animal models, i.e., rats, hairless guinea pigs, pigs and marmosets are presented for various routes of exposure, amongst others the highly relevant respiratory and percutaneous routes, using intravenous administration as the reference route. Despite the high reactivity of sulfur mustard the compound is remarkably stable *in vivo*, and the intact agent is highly persistent in the body of these animal species.

The analytical procedures to analyze intact sulfur mustard in blood and tissues of laboratory animals at toxicologically relevant levels are discussed. Also, the time-course of the predominant adduct to DNA and proteins in blood and various tissues is described, as well as methods for their analysis. The toxicokinetics in humans are unknown. However, data on sulfur mustard metabolites and adducts measured in humans accidently exposed to the agent are presented and discussed in the light of the observations made in laboratory animals.

Several knowledge gaps are being identified, and suggestions are made how to fill these.

Pathophysiology of Sulfur Mustard

Molecular toxicology of sulfur mustard poisoning

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The alkylating chemical warfare agent sulfur mustard (SM) is known to cause a plethora of clinical symptoms, e. g. blisters, erythema and inflammation after skin contact. Several hypotheses, aiming to describe the underlying molecular toxicology, were developed during decades of basic research. However, none of these hypotheses can be considered as all-encompassing. It is most likely that many mechanisms identified so far run in parallel and result in a complex pathophysiological pattern, which is still not entirely unraveled.

SM alkylates a multitude of cellular biomacromolecules, including DNA. As a consequence, DNA-repair processes are initiated which may affect cellular metabolism. Especially poly(ADP-ribose) polymerase (PARP) was described to be recruited after high SM exposures. Consumption of vital co-factors (e. g. NAD^+ and ATP) by PARP may result in necrosis, a fact that is reflected in the "Papirmeister-theory". As a consequence, inhibition of "PARylation" to counteract SM toxicity was performed in several studies, but results were not compelling. Besides necrosis, cell death triggered by both intrinsic and extrinsic apoptosis is frequently observed after SM exposure. Several attempts were made to prevent cell death, e. g. by addressing Fas-ligand or Fas-receptor or downstream caspases, but results were again unsatisfying. Extensive inflammation is another phenomenon after SM contact. Release of pro-inflammatory mediators such as IL-1ß, IL-6, IL-8 or TNF-α was demonstrated and linked to Nf-κB pathways. Thus, anti-inflammatory compounds such as COX-inhibitors seem to be useful in that context. Generation of radical species (ROS, RNS) after SM exposure is another frequently observed event. ROS scavengers had some protective effects on cytotoxicity and SM-induced affection of cell function, e. g. cell migration. However, the exact mechanisms is still unclear and thus further research is needed for a final evaluation.

This talk will summarize the most important aspects of the molecular toxicology of alkylating compounds and will outline current areas of research.

Acute and chronic pathologies in the corneal endothelium following ocular sulfur mustard exposure: towards a new understanding of corneal injury progression

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Sulfur mustard (SM) reactivity within biological tissues is rapid and irreversible, and consequently treatment strategies are focused on mitigating pathological host responses and promoting healing. However, therapies to preserve the corneal epithelium and reduce inflammation following ocular exposures have been ineffective in preventing chronic symptoms of mustard gas keratopathy (MGK). Hypothesizing that SM toxicity to the non-regenerative corneal endothelial cell layer at the posterior margin of the cornea represents a novel injury modality that explicates ocular SM injury progression, we characterized long-term effects of corneal exposure to SM vapor on the corneal endothelium in rabbits. Convergent methods revealed that ocular SM exposure causes acute endothelial toxicity and disruption of endothelial barrier function. Furthermore, MGK eyes exhibit long-term endothelial pathologies consistent with the endothelial-to-mesenchymal transition that is associated with endothelial failure. None of these features were observed in SM-exposed eyes that fully recovered. Importantly, the extent of endothelial toxicity was predictive of MGK development. The association of endothelial pathologies with MKG suggests that SM damage to corneal endothelial cells provides a novel mechanistic basis for ocular injury progression. Based on these findings, we hypothesize that (a) the severity of acute SM injury is determined by the degree of endothelial damage and (b) the efficiency of endothelial repair influences whether corneas resolve or develop MGK. These hypotheses explain the dose-dependence of corneal SM symptoms and predict that treatments to reduce endothelial toxicity or promote endothelial recovery will reduce

or eliminate acute and chronic manifestations of corneal SM exposure.

(Disclaimer: The views expressed in this abstract are those of the authors and do not reflect the official policy of the Department of Army, Department of Defense, or the U.S. Government. The experimental protocol was approved by the Animal Care and Use Committee at the United States Army Medical Research Institute of Chemical Defense and all procedures were conducted in accordance with the principles stated in the Guide for the Care and Use of Laboratory Animals and the Animal Welfare Act of 1966 (P.L. 89 - 544), as amended.)

Molecular targets for sulfur mustard leading to inflammation and tissue injury

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Sulfur mustard and nitrogen mustard (NM) are cytotoxic vesicants known to target the lung. They cause acute pulmonary injury which can progress to fibrosis. Our laboratories have been developing animal models of mustardinduced injury with the goal of assessing the role of macrophages in the pathogenic response. C57Bl6/J mice were treated with NM (0.08 mg/kg) or PBS control intratracheally. Bronchoalveolar lavage (BAL), alveolar macrophages and lung tissue were collected 3 d, 14 d and 28 d later. NM exposure induced time-related histopathologic changes in the lung including alveolar thickening, perivascular inflammation and bronchiolar epithelium hyperplasia, along with interstitial fibroplasia and fibrosis which were most notable at 14 d. Time related increases in enlarged foamy macrophages were also observed in the lung. This was associated with a rapid and persistent increase in total BAL cell, protein and phospholipid content, and surfactant protein (SP)-D levels. At 3 d and 14 d post NM, expression of iNOS and TGFbeta was also upregulated in lung macrophages. Flow cytometric analysis showed that macrophages accumulating in the lung 3 d and 14 d post NM were pro-inflammatory macrophages, while at 14 d and 28 d, macrophages expressed characteristics of anti-inflammatory macrophages. NM-induced structural and inflammatory changes were accompanied by increases in total lung resistance, tissue damping and elastance and decreases in compliance and static compliance. These data demonstrate that NM induces structural and functional changes in the respiratory tract of mice. Moreover, these changes are associated with a sequential accumulation of proinflammatory/cytotoxic and anti-inflammatory/wound repair macrophages in the lung.

Toxic Lung Injury

Molecular findings in toxic lung injury

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In recent years a paradigm shift regarding the proposed mechanisms of toxicity evoked. Hitherto, the toxic action has been mainly attributed to unspecific cell damage caused by reactions of the toxins with biomolecules. Recently, the identification of molecular down-stream effectors of redox signaling in cells was a major step forward in the mechanistic understanding of the action of toxic chemicals and will pave the way for more causal and specific therapeutic options.

Therefore, the involvement of Transient Receptor Potential (TRP) channels as direct or indirect chemosensors in the detection and action of toxins is an attractive hypothesis. Toxic inhalation hazards (TIH), including chemical warfare agents and cytostatic drugs like bleomycin are known to cause severe toxic lung injuries (TLI). However, as the exact pathomechanisms are still unknown in large parts, specific antidotes or other therapeutic measures are unavailable. While TRPC6 is indirectly activated by bleomycin and contributes to bleomycin-induced lung fibrosis, direct TRPA1 activation by sulfur mustard (bis-(2-chloroethyl) sulfide) increases intracellular Ca^{2+} concentration, phosphorylation of ERK1/2 (pERK1/2) levels, as well as induces translocation of the serum-response-element (SRE). Interestingly, antioxidants like N-acetyl-Lcysteine prevent TRPA1 activation. Moreover, different airway parameters like pulmonary blood flow, integrity of the epithelial lining, as well as mucociliary clearance are directly or indirectly controlled by TRP-channels.

This presentation will focus on the current state of knowledge regarding TRP channels and their potential role in TLI caused by TIHs. Specific TRP activating or inhibiting drugs may serve as novel therapeutic approaches for TLI in the future.

TRP ion channel inhibitors as countermeasures against chemical injuries

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The treatment of acute lung injury caused by exposure to reactive chemicals remains challenging due to the lack of mechanism-based therapeutic approaches. Recent studies have shown that Transient Receptor Potential Vanilloid 4 (TRPV4), an ion channel expressed in pulmonary tissues, is a crucial mediator of pressure-induced damage associated with ventilator-induced lung injury, heart failure and infarction.

We examined the effects of two novel TRPV4 inhibitors in mice exposed to chlorine gas, a severe chemical threat with frequent exposures in domestic and occupational environments and in transportation accidents. Post-exposure treatment with a TRPV4 inhibitor suppressed pulmonary inflammation by diminishing neutrophils, macrophages and associated chemokines and cytokines, while improving tissue pathology. These effects were recapitulated in TRPV4-deficient mice. TRPV4 inhibitors also inhibited vascular leakage, airway hyperreactivity and increase in elastance, while improving blood oxygen saturation.

In chlorine-exposed domestic pigs, a TRPV4 inhibitor improved oxygenation and respiratory physiological parameters and reduced total BALF cell and neutrophil counts. Vascular leakage and edema and pro-inflammatory cytokine markers were decreased and histopathological scores improved.

Taken together these results suggest that TRPV4 inhibitors hold promise as new countermeasures for the treatment of chlorine-induced lung injury.

Animal modelling of acute and long-term effects of sulfur mustard inhalation, and potential therapeutic interventions

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Rationale: Inhalation of sulfur mustard (SM) can have debilitating acute and long-term pulmonary consequences, such as acute airway obstruction by fibrin casts, as well as chronic lung diseases such as bronchiolitis obliterans (BO) and parenchymal fibrosis (PF). The underlying pathogenesis of disorders after SM inhalation is not clearly understood, resulting in a paucity of effective therapies. *Methods:* Yorkshire swine (~45 kg) and Sprague-Dawley rats (~275 g) were used to model acute and chronic effects, respectively, of SM inhalation. Animals were sedated, intubated, and exposed to SM vapor. Swine were monitored continuously for 24 h. Rats were monitored daily for 28 days, with endpoints of survival, oxygen saturation, respiratory distress, arterial blood gas changes, blood chemistry, weight loss, changes in histopathology, pulmonary function testing, and assessment for TGF- β and PDGF. Nintedanib, an antifibrotic drug, was given once daily in rats for 28 days, and improvements assessed.

Measurements and Main Results: A successful swine model was created to mimic acute high dose SM inhalation injury of humans. In rats, survivors of acute pulmonary injury developed respiratory distress at ~14 days after exposure, with progressive hypoxemia, retractions, and weight loss. Histopathology confirmed the presence of both BO and PF, gradually worsening with time. Pulmonary function testing demonstrated a time-dependent increase in lung resistance, and a decrease in lung compliance. Levels of TGF-β and PDGF in lungs were elevated by days 21, in both lung tissue and bronchoalveolar lavage fluid (BALF). Nintedanib treatment improved some endpoints, particularly those related to PF. *Conclusion:* Swine is a good model to study human disease, with humanrelevant endpoints. In rats, time-dependent development of BO and PF occurred in the lungs after SM inhalation, and the elevated levels of TGF-β and PDGF suggest involvement of these pro-fibrotic pathways in the aberrant remodeling after injury. Nintedanib showed promise in improving relevant long-term outcomes after SM inhalation.

Activation of chemosensing TRPA1-channels by Lewisite

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The human transient receptor potential ankyrin 1 (hTRPA1) ion channel is responsible for the irritation and pain associated with exposure to many reactive chemicals resulting in blepharospasm, lacrimation, rhinorrhea, tightness of the chest, coughing, sneezing and increased respiratory tract secretions. Here we show that for the vesicants Lewisite and sulfur mustard, their activity at this channel can be linked to irritancy in man. A cell-based assay (HEK PhotoScreen™ TRPA1 cell line) was used to examine activity by measuring $[Ca^{2+}]_i$ following vesicant additions to activate the channel. hTRPA1 was potently activated by Lewisite 1 (EC50 2 μM) and Lewisite 2 (EC50 0.03 μM) but not by Lewisite 3. Sulfur mustard, used over the same concentration range (up to 30 μM), failed to activate the hTRPA1 channel. Lewisites 1 and 2 did not generate a response in wild-type HEK cells and the responses in hTRPA1 cells were completely inhibited by the antagonist A967079 and, for Lewisite 1, by British Anti-Lewisite (Lewisite 2 was not tested). Activity at the hTRPA1 channel can explain the observations in man that exposures to the Lewisites, but not to sulfur mustard, are associated with almost immediate irritation and that Lewisite 2 causes much more intense irritation than does Lewisite 1, with Lewisite 3 being non-irritant.

Innovative Approaches in Sulfur Mustard Research

The role of miRNAs in sulfur mustard toxicology

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After contact with the skin, sulfur mustard (SM) induces blister formation and long-term complications such as impaired wound healing. Previous own studies demonstrated that exposure of normal human epidermal keratinocytes (NHEK) with SM resulted in reduced proliferation, premature differentiation and a restricted functionality of hypoxia-mediated signaling in the cells. Hence, we hypothesized that microRNAs (miRNAs) might be involved in these mechanisms. miRNAs are short non-coding RNA molecules with important roles as posttranscriptional gene regulators in cells. miR-203 and miR-210 are known to influence epidermal cell function in normoxia and hypoxia, respectively. Our recent studies revealed that SM significantly upregulated the expression of miR-203 in NHEK when cultivated under normoxic and hypoxic conditions. In contrast, SM had no effect on miR-210 under normoxia. However, miR-210 levels were greatly increased in NHEK when grown in hypoxia, as to be expected, but were further elevated upon exposure of the cells to SM. Next, we studied the effect of decreasing miR-203 and miR-210 activities by transfection of these cells with antisense oligonucleotides that specifically block miR-203 and miR-210, respectively. In normoxia and hypoxia, inhibition of miR-203 attenuated the SM-induced impairment of metabolic activity and proliferation, and counteracted SM-promoted keratin-1 expression in these cells. Consistent ameliorating effects on dysregulated metabolic activity, proliferation and keratin-1 expression in SM-treated NHEK were obtained upon inhibition of miR-210 in these cells grown in hypoxia. Our findings provide evidence that miR-203 and miR-210 are key regulators in normal and SM-impaired keratinocyte functionality, and suggest usefulness of miRNA inhibitors for target-directed therapeutical intervention to improve re-epithelialization of SM-injured skin.

Sulfur mustard and epigenetics

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Sulfur mustard (SM) and nitrogen mustards (NM) are alkylating agents that affect DNA and other biomacromolecules. Long-term health effects include skin xerosis, pigmentation disorders, vessel abnormalities like hemangioma but also malignant neoplasms which can occur even years after a single exposure. The molecular toxicology is still unclear and subject of current research. Besides structural DNA alterations, epigenetic DNA modifications that may persist over decades are discussed to mediate SM-induced long-term effects. To address this question, human endothelial were exposed to SM or NM (chlorambucil) and epigenetic modulations (5-mC, 5-hmC, histonemodifications) were investigated either over time (1 - 4 d) or over several cell passages. Moreover, influence of inhibitors of epigenetic modulatory enzymes (5-Aza-2'-deoxycytidine (DAC), procaine) on DNA methylation was assessed. In addition, effects on fundamental cell functions were evaluated. Complex epigenetic modulations after SM or NM exposure became evident that persisted over time and cell generations. DNA hyper-methylation (5-mC) but also increase of 5-hmC was observed. Regarding histone modifications, both deacetylation and methylation were evident which could be prevented using DAC or procaine. Remarkably, DAC also counteracted alkylating compound induced toxicity.

In summary, epigenetic modifications could be one reason for long-term tissue damage after SM or NM intoxication and can explain pathophysiological findings occurring even years after exposure. Addressing these effects may represent a new, but promising therapeutical approach counteracting SMinduced long-term health effects. *In vivo* studies should be conducted to validate our *in vitro* findings.

Stem cells under the influence of sulfur mustard

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Even 100 years after the first use of sulfur mustard (SM), no antidote is available for the therapy of poisoned victims and treatment is symptomatically. As a promising therapy option, skin graft has been shown, but this is not always applicable. Stem cells offer an alternative and innovative approach. Mesenchymal stem cells (MSC) are important for the regeneration of wounded skin. Essential for a MSC driven wound regeneration is active migration into the wounded area. SM wounded skin shows chronic wounds with an impaired wound healing for weeks till month. Altered migratory capacity of MSC is typical for chronic wound healing disorders. Therefore, we investigated the effects of SM on MSC. It could be shown that MSC tolerate a high level of SM but their properties of migration that are necessary for their regenerative influence is decreased under influence of SM.

Induced pluripotent stem cells (iPS) are another promising approach which enables autologous tissue replacement. For reprogramming key pluripotency factors were added that induce and coordinate the reprogramming.

To this end episomal plasmids were used. This method was selected to apply the procedure of iPS generation to various cell lines of various patient materials. Indeed, the basic method of generating iPS has been established successfully.

Using this technique, it could be shown that the above described reduction of migratory capacity can be compensated in part by the addition of cytokines. That compensation most possibly provides an option to resolve the problem of disturbed wound healing.

Moreover, our current results will help to understand the relationship between alkylating agents and stem cells and thus may also give guidance in the future perspective for the therapeutic use of MSC in patients suffering from SM poisoning.

Immediate neuro-biological effects of sulfur mustard in insects

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Introduction: Sulfur mustard (SM) induced toxic effects appear with a characteristic clinical latency which is assumed to be caused by unspecific cell damage, e. g. DNA alkylation. However, SM was proven to immediately activate chemosensory TRP ion channels *in vitro* which could theoretically result in acute cellular effects. In line with this hypothesis, it was recently demonstrated that cockroaches exhibit rapid clinical symptoms and death after exposure to alkylating compounds.

Methods: The Argentinian wood cockroach (*Blaptica dubia*) was used as an *in vivo* model to investigate immediate clinical effects of alkylating compounds. Animals were exposed to various concentrations of bi- and monofunctional alkylating compounds (SM, NM, CEES). Biological behavior (e. g. antenna pull back reflexes, escape behavior) was monitored for 1 h and evaluated by calculating a disability score, introduced especially for our model. Neuronal signal transmission of sensory signals was examined by electrophysiological recordings of extracellular field potentials.

Results: Neat SM, but not CEES induced an instantaneous pull-back reflex after exposure. Subsequently, an escape reaction was observed which was characterized by persistent and high-frequency leg kicks. Surprisingly, the CEES-induced symptoms were more strikingly compared to SM. NM had no effects during the observation period. Measurements of the extracellular field potential revealed a fast and concentration-dependent signal transmission from the antennae to the ganglion innervating the legs.

Conclusion: Our results prove instant toxic effects of SM in *Blaptica dubia* which cannot be explained by DNA alkylation. Thus, the old dogma that DNA alkylation with impact on protein expression is the cause for SM toxicity has to be reappraised.

Air-liquid interface exposure systems for the *in vitro* investigation of pulmonary toxicity of chemical warfare agents

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Inhalation of chemical warfare agents can cause severe acute health effects including pulmonary dysfunction due to e. g. inflammation or loss of epithelial or endothelial integrity. The underlying molecular toxicology is still mostly unknown and primarily studied using animal models. However, ethical issues question the use of *in vivo* experiments. *In vitro* studies that can be conducted in first instance represent a valuable alternative. To mimic the physiological situation as close as possible, such *in vitro* models have to meet certain demands. The most critical aspect is the mode of exposure: submerse exposure of pulmonary cells, which is probably prevalent in most studies, is not considered adequate because an alternation of chemical and toxicological properties might occur. Exposure of cells at the air-liquid-interface (ALI) avoids this artificial exposure conditions and is preferable. The talk will give an overview about available *in vitro* ALI exposure systems and present the properties of the respective systems. Moreover, our own results using the CULTEX® Radial Flow System (RFS), a specially designed modular *in vitro* exposure system that enables a homogenous exposure of lung cells to airborne particles at the ALI, will be presented. The CULTEX® RFS was modified by exchanging the CULTEX® dust generator with different aerosol generators. The initially favored thermal evaporization was found inappropriate, as most agents, including sulfur mustard (SM), are diluted in organic and flammable solvents, thus presenting the risk of ignition or explosion. Two nebulizer systems (PariBoy and eFlow) were tested as alternative. First preliminary experiments with ethanol indicated that the eFLOW seems most suitable for SM-exposure of cells at ALI. A proof of principle study using SM as toxic compound is currently in process.

Findings from Basic Research and in vivo Models

Toxicology and medical treatment of tear gases

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Tear gas agents (TGAs) are pain-inducing aerosols. TGAs are used to deter people without inflicting injury. Most TGAs are lipophilic with electrophilic sites. The electrophilic sites of TGAs activate TRPA1 ion channels via interactions with cysteines. TRPA1 channels are expressed on A-delta and C-fiber nociceptors of trigeminal and other somatosensory nerves that innervate the facial mucosa. Activation of TPRA1 channels carries Ca^{2+} and Na^{+} into the processes, which results in: 1) action potential propagations that signal for pain perception and protective autonomic reflexes, and 2) release of pro-inflammatory peptides at the site of insult. TRPA1 mediated pain responses (or nociceptive behaviors in rodents) can often be blunted with local anesthetics or reagents that inhibit TRPA1 activation/channel opening. TRPA1inhibitors are effective in preventing TRPA1-mediated protective airway responses. Unfortunately, TGAs can produce injuries. TGAs can produce severe effects in people with pre-existing conditions of the: 1) exposed areas, such as dermatitis, 2) TRPA1-expressing neurons, such as optic hyperalgesia, or 3) protective autonomic responses, such as asthma. Furthermore, repeated or severe exposures to reactive chemicals, such as TGAs, are associated with hyper-sensitivity conditions including asthma and dermatitis. TRPA1-induced neurogenic inflammation maybe involved in exacerbating these conditions. Chemical burns of the skin, eyes, mouth and airways due to TGAs-electrophilic reactions have also been reported. To prevent burns, soap and water are fairly effective at decontaminating and inactivate TGAs. Certain TGAs induce pain by capsaicin, which activates a different ion channel, TRPV1, on nociceptors. Many of the actions and treatments for TGAs that activate TRPV1 are similar to those for TGAs that activate TRPA1.

Toxic effects of cutaneous phosgene oxime exposure

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Stockpiled during World War II, Phosgene oxime (dichloroformoxime, CX; Cl_2CNOH) is a potent chemical weapon and poses a threat of delivery by itself or with other chemical agents to cause surprisingly prompt incapacitation and death. CX is also categorized as a vesicating agent; however, this halogenated oxime does not form actual blisters but is an urticant, nettle or a corrosive agent. In both liquid and vapor forms, CX causes more severe damage than other vesicants due to its fast penetration, immediate pain and tissue destruction. Hence, the acute effects of CX following its cutaneous exposure in SKH-1 hairless mice were studied with a goal to help establish a relevant CX-induced injury model. Topical cutaneous exposure to CX vapor caused blanching of exposed skin with an erythematous ring, necrosis, edema, mild urticaria and erythema within minutes after exposure out to 8 h post-exposure. These clinical skin manifestations were accompanied with increases in skin thickness, apoptotic cell death, mast cell degranulation, myeloperoxidase activity indicating neutrophil infiltration, p53 phosphorylation and accumulation, and an increase in COX-2 and TNFα levels. Topical CX exposure also resulted in the dilatation of the peripheral vessels with a robust increase in RBCs in vessels of the liver, spleen, kidney, lungs and heart tissues. These events could cause a drop in blood pressure leading to shock, hypoxia and death. This is the first report on effects of CX cutaneous exposure, which could help design further comprehensive studies evaluating the acute and chronic skin injuries from CX topical exposure and elucidate the related mechanism of action to aid in the identification of therapeutic targets and mitigation of CX cutaneous exposureinduced morbidity.

Acute systemic toxicity of sulphur mustard in anesthetized swine

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Recent Iranian publications have highlighted the long-term and chronic effects of sulphur mustard (H) in casualties of the Iran-Iraq War. These effects include cancers, as well as immunological, respiratory, dermal and neurological symptoms. An instrumented anaesthetized domestic swine model was used to investigate the short-term systemic effects of H. Animals (20 kg) were injected intravenously with 5, 12 or 20 µL of H in a saline solution and physiology and clinical chemistry were closely followed for the following 6 hours along with samples for quantification of blood and tissue levels. A subset of animals were subsequently revived and observed for an additional three days. During anaesthesia, mean arterial pressure was reduced, heart rate increased and some respiratory parameters appeared to be changed in a dose-dependent manner. There were no consistent changes in the haemotological, biochemical or electrolyte parameters examined pre- or post-anaesthesia. Observations made during post-anaesthetic recovery showed dose-dependent trends in neurological and gastrointestinal endpoints, including weight loss. Lower limb weakness was observed at the highest dose. Histopathological evaluation of tissues collected showed dose-dependent changes in the GI tract and the lymphoid system. Potentially significant lesions of the nervous system were observed. Necrosis of the leptomeninges was noted, while under electron microscopy a dose-dependent unwinding of the myelin sheath of the tenth cranial nerve was documented.

Use of novel microsampling devices for detection of sulfur mustard albumin-adducts in dried plasma

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Biomedical verification of poisoning by sulfur mustard (SM) using modern instrumental analysis may require sample transport from a crisis region to specialized laboratories. Liquid and frozen plasma is categorized as infectious material (UN3373). Therefore, its shipment by air, sea or on the street is subject to strict international regulations dictating a complex three-fold packaging causing high costs. In the recent past it was found that observing these rules represents an essential challenge sometimes constraining prompt and adequate sample analysis. In contrast, dried blood/plasma samples are not categorized as infectious material anymore and may thus be sent in simple envelopes by post. Using this dried blood spot technique novel procedures were developed allowing detection of SM-albumin-adducts (SM-HSA-adducts). Modern blood/plasma microsampling devices introduced on the market a few years ago (Mitra devices and Noviplex DUO cards) were tested as well as more conventional and wellknown filter paper. Dried plasma was dissolved in buffer prior to proteolysis of the SM-HSA-adduct and subsequent liquid chromatography tandem-mass spectrometry for qualitative biomarker detection. Storage stability of adducts in dried plasma was found at ambient temperature and relative humidity of different climates corresponding to Central Europe (temperate), Middle East (hot and dry) and the tropical equatorial regions (hot and humid). Selected microsampling devices were shown to enable precise and accurate as well as reliable adduct detection thus underlining their value for future studies and military missions. Noviplex DUO cards were successfully applied to whole blood samples of swine documenting SM poisoning after 2 months of shipping and storage at ambient temperature.

Nerve Agent Poisoning

Medical challenges in the treatment of organophosphorus poisoning

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The deployment of the nerve agent sarin in the Syrian civil war in 2013 with several thousands dead or wounded underlines the persistent threat emerging from organophosphorus compounds (OP). All OPs exert their acute toxic effects primarily by covalently binding to the pivotal enzyme acetylcholinesterase (AChE), where phosphylation of the catalytic serine residue renders the enzyme inactive and may finally result in respiratory arrest and death due to excess of acetylcholine.

Current established medical countermeasure procedures date back to the 1950s and comprise an oxime as causal antidote to reactivate the phosphylated AChE. However, several classical nerve agents as soman and tabun are resistant towards this standard treatment. Numerous derivatives of classical nerve agents were described in the open literature with in part insufficient data regarding detection, toxicity and efficacy of oxime treatment. Additionally, less toxic OP pesticides are widely distributed in agriculture and 260,000 death per year occur due to mostly suicidal poisoning or accidental exposure. OP pesticides could be used as nerve agents by disseminating larger amounts to compensate their lower toxicity.

As 60 years of oxime research did not result in substantial progress even for classical nerve agents, the research focus switched from oximes to scavengerbased concepts or modulation of nicotinic receptors. (Bio-)Scavengers are administered to detoxify nerve agents in the systemic circulation prior to distribution into target tissues. Recent progress in the design of scavengers with encouraging results in *in vivo* studies supports this concept. Another concept under investigation is the allosteric modulation of nicotinic receptors with bispyridinium compounds showing promising results *in vitro* and *in vivo*.

Efficacy of phosphotriesterases in case of V-agent poisoning

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The recent use of sarin against Syrian civilians as well as the potential capability of synthesis by terrorists underlines the

threats of nerve agents. Standard treatment protocols with atropine and an oxime have a limited effectiveness against different nerve agents. An innovative complementary approach is the development of phosphotriesterases (PTE) as catalytic bioscavengers. In collaboration with the Weizmann Institute of Science, Israel, an enzyme mutant with a considerable activity against VX *in vitro*, C23, was examined as post-exposure treatment in VX-poisoned (2 × LD50) and anaesthetized guinea pigs. The enzyme impeded complete inhibition of acetylcholinesterase (AChE) in brain tissue, almost prevented systemic symptoms and resulted in survival of all treated animals. This study has been the first proof of concept of a VX treatment with a PTE *in vivo*. Intraosseous (i.o.) injections are increasingly important in military and emergency medicine. Consequently, this route of application was chosen in a subsequent study with a different PTE mutant as post exposure therapy in VX poisoned guinea pigs. The i.o. injection was compared with intravenous (i.v.) injection and resulted in a virtually identical PTE concentration and survival of all animals. However, treatment with the presently available PTE mutants requires rather high enzyme doses in the range of 2 - 5 mg/kg. In addition, these PTE mutants exhibit substantial substrate specificity. Therefore, further research is urgently needed and should be focused on the design of PTE mutants with a considerably higher catalytic activity and broader substrate spectrum.

Low molecular weight scavengers for V-type nerve agents based on cation-binding macrocycles

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The administration of scavengers that rapidly detoxify neurotoxic organophosphates (NOP) is a promising therapeutic approach for the treatment of nerve agent poisonings. The currently most potent NOP scavengers are based on proteins, but several aspects still need to be clarified before such bioscavengers can be used in practice. Synthetic scavengers might not have these drawbacks and therefore represent interesting alternatives.

Development of synthetic scavengers mainly concentrated on cyclodextrins so far but compounds that detoxify V-type nerve agents have remained elusive. A possible explanation could be that V-type nerve agents are poor substrates for cyclodextrins because of their charged and therefore polar nature at physiological pH. To test this assumption, we recently started to investigate macrocyclic compounds as scaffolds for nerve agent scavengers, which are known to interact with cations in water. We found in this context a sulfonated calixarene containing a substituent with a hydroxamic acid group that detoxifies VX and structurally related V-type nerve agents with, for a synthetic compound, so far unmatched activity. The half-live of VX detoxification in the presence of this calixarene amounts to ca. 3 min in TRIS-HCl puffer at pH 7.4 and 37 °C, for example. We attribute the observed detoxification ability to the presence of the calixarene ring, which introduces a millimolar affinity for positively charged organophosphates, in combination with the proper arrangement of the hydroxamic acid group. This calixarene thus represents a highly promising lead structure for novel antidotes. In my contribution, I will present our concept of scavenger development and discuss the obtained results.

Nicotinic acetylcholine receptor modulators

K.T. Wanner, G. Höfner, S. Rappenglück, S. Sichler, T. Wein

Department of Pharmacy, Center of Drug Research, LMU, Munich, Germany

Organophosphorus nerve agents (OP) cause their toxic effects by an irreversible inhibition of the enzyme acetylcholinesterase. This results in an accumulation of the neurotransmitter acetylcholine (ACh) and subsequently in an overstimulation and in a desensitization of nicotinic acetylcholine receptors (nAChR) with an ultimate suppression of neuronal signals. It has been shown that these pathologic conditions of desensitization of the nAChR could be at least partially reversed and the muscular strength could be restored by a series of symmetrically substituted bispyridinium compounds. The bispyridinium salt MB327 is considered as one of the most potent compounds out of this series known so far thus being a promising starting point for the development of new and more potent positive allosteric inhibitors of type II at nAChR.

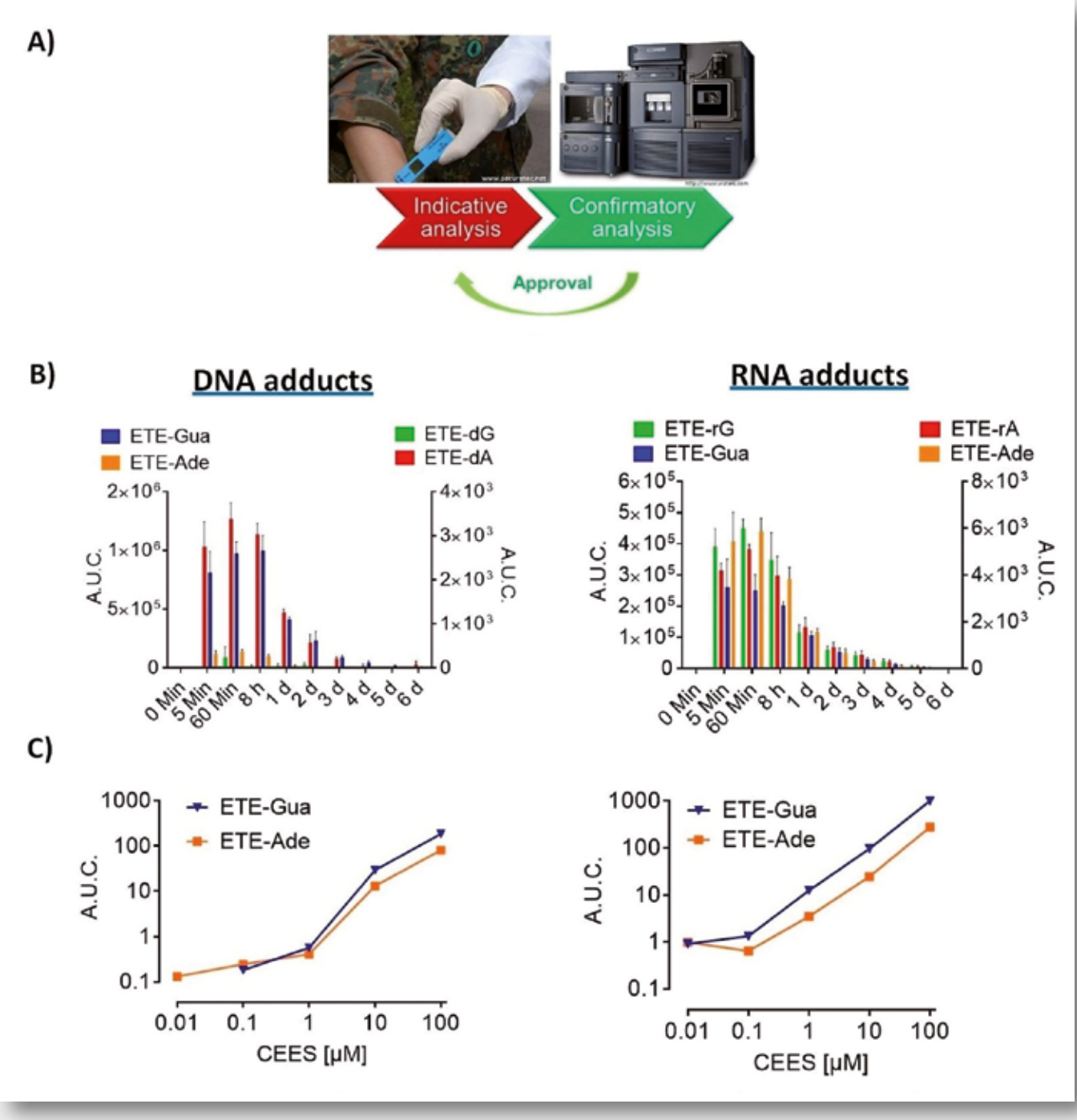
Using MB327 as lead compound, we conducted an integrated medicinal chemistry study to develop new bispyridinium based nAChR "re-sensitizers". For the rational design of such compounds, ligand and structure based models have been established. New compound candidates have been synthesized employing newly developed synthetic strategies providing an efficient and flexible access to a large number of differently substituted compounds. For the determination of the binding characteristics, a new MS based binding assay ("MS Binding Assay") based on a native, not radioactive labelled marker has been established.

(This project is funded by the German Ministry of Defence (E/U2AD/CF514/DF561))

Poster Award: 1st Place

Tabea Zubel (Konstanz, Germany):

Development of a mass spectrometric platform for the quantitation of mustard-induced nucleic acid damage



A mass spectrometric platform for the quantitation of mustard-induced nucleic acid damage

A) Guidelines for the GTFCh for forensic-toxicological analyses: A combination of indicative, based on antibody detection, and confirmatory analysis, based on mass spectrometry, provides data that can bei used as evidence in court.

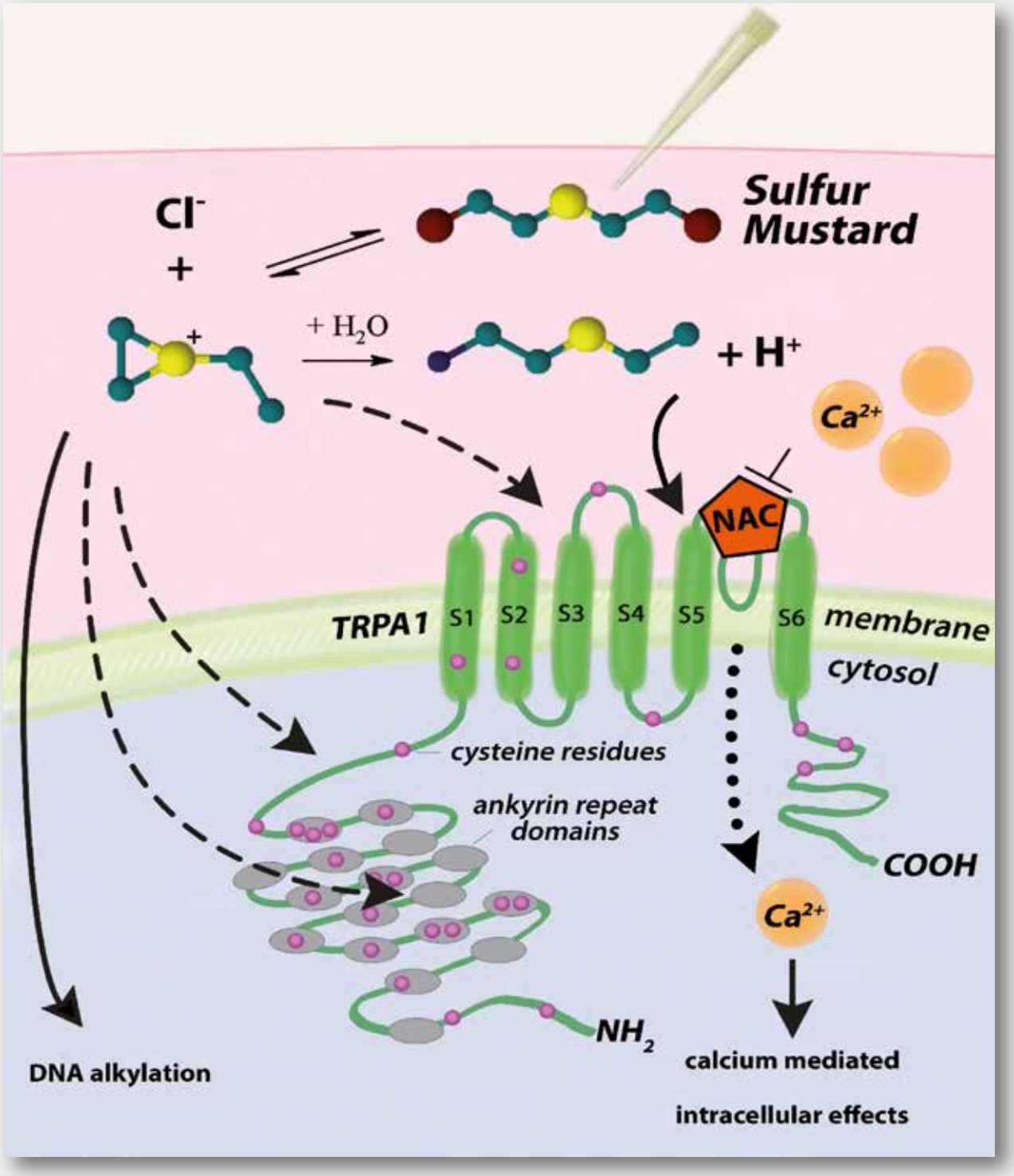
B) Time series of CEES adducts in HaCaT keratinocytes: HaCaT cells were treated with 100 µM CEES for 10 min and further incubated for periods as indicated. Sustained adduct stabilities were detected in DNA as well as RNA adducts at least for 6 days after induction.

C) Absolute DNA Adduct quantification by isotope dilution mass spectrometry (ID-UPLC-MS/MS): Human peripheral blood mononuclear cells (PBMC) (left part) or whole blood samples (right part) were treated with CEES for 1h. The lower limit of detection (LOD) was found to be < 1 fmol for both ETE-Gua and ETE-Ade.

Poster Award: 2nd Place

Bernhard Stenger (Munich, Germany):

Effect of N-acetylcysteine and glutathione on alkylating agents-induced TRPA1-channel activation

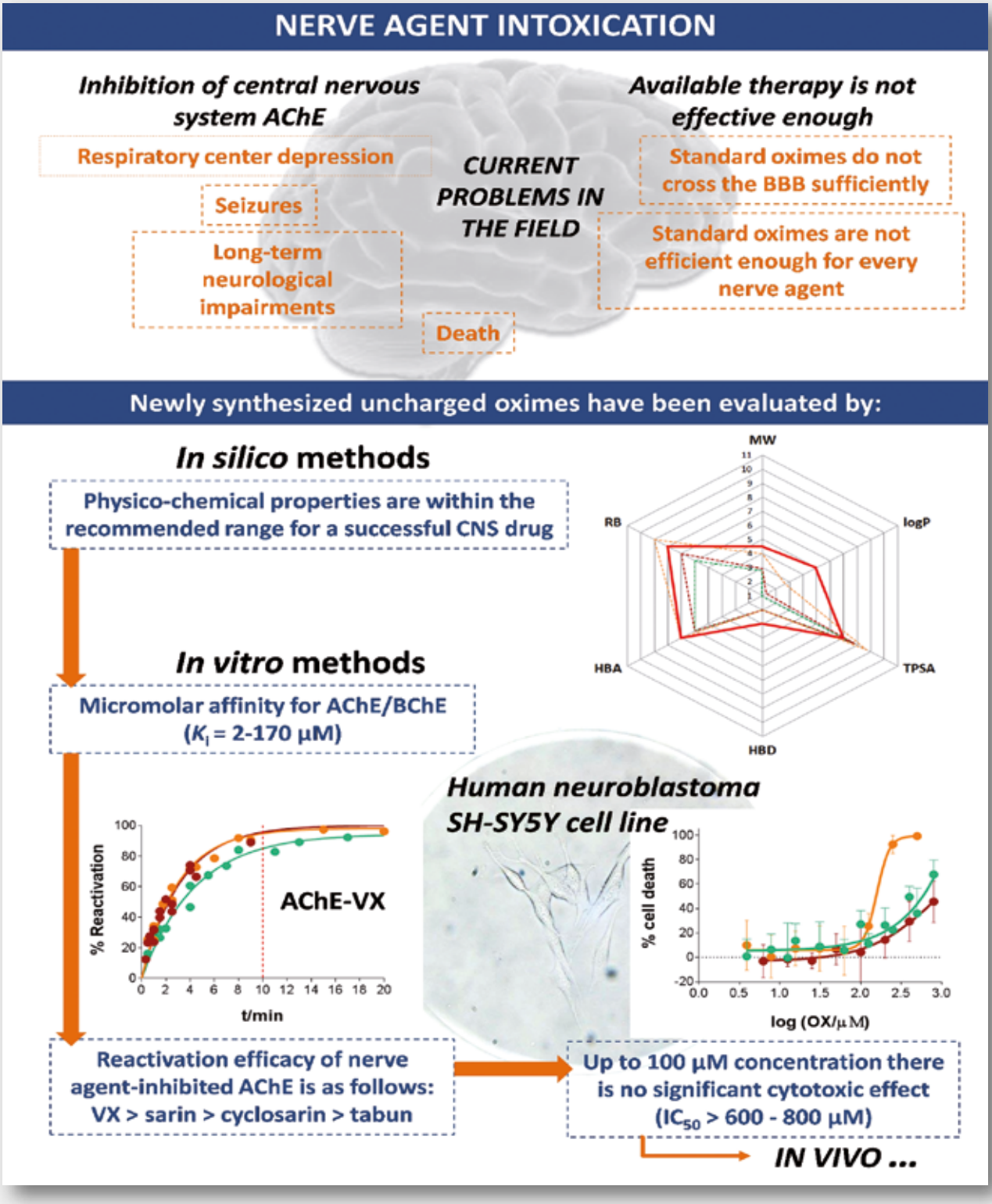


Intracellular cysteine residues have been discussed as targets for covalent modifications, including alkylation, resulting in TRPA1 activation. Alkylation of DNA and proteins by SM and related compounds is well known. Thus, alkylation of nucleophilic amino acid residues, including intracellular cysteine residues of TRPA1, may also occur. Proton-induced activation of TRPA1, associated with the transmembrane domains 5 and 6, has also been discussed. Thus, proton release during hydrolysis of SM may contribute to a direct TRPA1-mediated calcium influx. However, the calcium influx induced by SM is more intense and sustained than the HCl induced increase in $[Ca^{2+}]_i$. Specific TRPA1-blockers and also pre-incubation of our cells with the antioxidant N-acetylcysteine prevented SM-induced TRPA1 activation. Further experiments have to unravel the exact mechanism.

Poster Award: 3rd Place

Tamara Zorbaz (Zagreb, Croatia)

New uncharged potent reactivators of AChE and BChE inhibited by nerve agents



New uncharged oximes have been evaluated by in silico and in vitro methods. They show promising in vitro reactivation potential of acetylcholinesterase (AChE; EC 3.1.1.7) inhibited by different nerve agents. Good permeability of novel oximes through the blood-brain barrier (BBB) is expected due to favourable physicochemical properties. Novel reactivators are not cytotoxic for the possible target cells (i.e. human neuroblastoma cell line SH-SY5Y) at both the active and in vivo achievable concentrations. Further experiments with novel oximes will include in vivo evaluation of their therapeutic potenial.

Poster Presentations

Investigation of sulfur mustard, polysulfide analogues and reactive intermediates from Levinstein mustard by density functional theory (DFT)

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Recently, a OPCW Fact Finding Mission (FFM) was able to confirm "with utmost confidence" that at least two people were exposed to sulfur mustard (HD) in the town of Marea in Syria during an attack on 21 August 2015 allegedly carried out by the so called "Islamic State". The OPCW has also recently worked with Iraqi authorities leading to the confirmation of the use of sulfur mustard in Northern Iraq. As thiodiglycol, the main precursor for the production of pure sulfur mustard, is a highly regulated chemical, non-state actors might turn to alternative production methods that result in an impure but still highly toxic form of "crude" sulfur mustard. One of these alternative methods is the Levinstein Process.

The two main impurities in Levinstein Mustard are bis(2-chloroethyl)disulfide (HS2) and bis(2-chloroethyl)trisulfide (HS3). HS2 and HS3 lead to significant amounts of degradation/reaction products in the environment resulting in unique chemical signatures. Therefore, the reactivity and potential reaction pathways of HS2 and HS3 are of significant importance but were not subject of intense study since the late 1940s. In addition, the toxic properties of HS2 and HS3 are also of special interest. Sulfur mustard itself is a strong vesicant agent and mutagen. Data regarding the toxicity of HS2 and HS3 is rare but the existing information indicates significantly reduced vesicant properties.

We use quantum chemical methods employing Density Functional Theory to determine reaction pathways and energy barriers for HD, HS2 and HS3 with the nucleophiles water, hydroxide and methylthiolate (as a simple model for cysteine thiol). The data is able to explain the different reactivities of HD vs. HS2 and HS3 and might also explain their significantly altered toxicity.

Interference of alkylating compounds with fluorescent DNA-labeling

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Introduction: The chemical warfare agent sulfur mustard (SM) and similar compounds, e.g. nitrogen mustards, are known to induce DNA alkylation. Covalent DNA modifications of guanine residues can be visualized by specific antibody stainings using e.g. immunofluorescence techniques. Total DNA amount, e.g. determined by DNA-fluorescence labeling, is routinely used for normalization. However, we observed an influence of SM on fluorescent DNAlabeling. This phenomenon was investigated in detail in the presented study.

Methods: DNA of human keratinocytes (HaCaT cells) was extracted, stained with various fluorescence dyes, i.e. ethidium bromide (EthBr), and subsequently exposed to different S- and N-alkylating agents (e.g. SM, CEES, NM). Fluorescence was measured using a microplate reader. Mass spectrometry and in-depth fluorescence analysis were performed to detect potential modifications of EthBr by SM. DNA was digested using various restriction enzymes. Moreover, buffers were used instead of pure water (AQ) in additional experiments.

Results: Alkylating compounds dose-dependently decreased fluorescence of EthBr-stained DNA. Direct interference of SM with EthBr was excluded by MS and fluorescence measurements. DNA crosslinks were assumed to cause the loss of fluorescence. In line with that hypothesis, DNA cleavage by restriction enzymes restored fluorescence. However, protons, released during alkylation or hydrolysis of SM, were finally found to quench the fluorescence signal. This was prevented by using appropriate buffers instead of AQ in our experiments. *Conclusion:* Fluorescence of EthBr-stained DNA was quenched by alkylating compounds in pure water. The use of appropriate buffers prevented the decrease of fluorescence. Thus, we highly recommend using buffers in such experiments.

In vitro response of human lung cells to sulfur mustard exposure

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Sulfur mustard (bis(2-chlorethyl) sulfide) is a highly poisonous, chemical warfare agent that has been used in past in several armed conflicts. The exact mechanisms of its toxic effects, which lead to cell death, have been intensively studied. Lungs are one of the possible target organs.

In our present work, we compared time-dependent changes in sulfur mustard exposed adult human lung fibroblasts NHLF and lung alveolar cell line A-549. Mitochondrial membrane potential ($\Delta\psi m$, flow cytometry), apoptosis (flow cytometry), cell viability (MTT assay, Calcein-AM assay and xCELLigence – real-time cell analysis) and cell cycle distribution (flow cytometry) were studied.

We observed significantly decreased mitochondrial membrane potential and subsequent induction of apoptosis correlating with decreased cellular viability in the sulfur mustard exposed cells. In low concentrations, sulfur mustardinduced S-phase cell cycle arrest, on the other hand, high concentrations, cell cycle phase distribution of sulfur mustard exposed cells resembled cell cycle phase distribution of control group, which implies nonspecific cell cycle inhibition.

(Acknowledgments: This work was supported by a long-term organization development plan "Medical Aspects of Weapons of Mass Destruction" of the Faculty of Military Health Sciences, University of Defense.)

Anti-apoptotic and anti-inflammatory effects of berberine in sulfur mustard exposed keratinocytes

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Introduction: SM is a chemical warfare agent that had repeatedly been used in 20th century conflicts. Alleged use in ongoing 21th century conflicts has been reported. After contact, SM primarily affects eyes, lungs and skin. Main associated skin symptoms, i.e. erythema, skin blistering and ulceration, are accompanied by severe inflammation. Berberine (BER), a secondary metabolite produced by various medicinal plants, showed many beneficial antiinflammatory effects. This promising finding initiated an *in vitro* screening using SM-exposed keratinocyte models.

Material & Methods: Mono-cultures (MoC) of HaCaT cells and co-cultures (CoC) of HaCaT and THP-1 cells were used as *in vitro* models. As molecular markers for anti-inflammatory response we quantified interleukin 6 (IL-6) and interleukin 8 (IL-8) levels 24 hours after SM exposure. Moreover, both necrotic and apoptotic cell death were assessed by the combination of a cell death detection ELISA and a cytotoxicity bioassay.

Results: BER showed cytoprotective effects by reducing apoptosis up to 60 % compared to sham-treated controls. Necrosis remained mainly unaffected, with limited beneficial results in HaCat MoC. Determination of pro-inflammatory cytokines showed that BER has the potential to reduce both, IL-6 and IL-8 levels significantly. Especially in CoC, BER treatment had pronounced impact on cytokine levels.

Conclusion: Our *in vitro* results indicate that BER might be a promising substance counteracting SM-induced cell death and inflammation. Future research using *in vivo* models has to verify these findings, in order to prove BER as beneficial therapeutic after SM exposure.

Modulation of Nrf2 and NF-κB pathways by methylbardoxolone (CDDO-Me) in sulfur mustard and radiation exposed keratinocytes

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Introduction: Recent events reporting the abusive use of sulfur mustard (SM) during the sustained conflict in the Middle East underline the need of an effective therapy which is lacking up to date. SM is known to affect cellular pathways associated to inflammatory processes, like the Nrf2- and NF-κBpathway. Both signaling cascades were found to be modulated by CDDO-Me. Therefore, we investigated the potential of CDDO-Me as therapeutic substance after SM exposure *in vitro*. As the clinical picture of SM poisoning and radiation exposure are similar (e.g. severe inflammation and impaired wound healing) and CDDO-Me is already discussed to have beneficial effects in radiations scenarios, we investigated the effect of CDDO-Me on radiation-exposed keratinocytes in addition.

Materials & Methods: NHEK (Normal Human Epidermal Keratinocytes) were either pre-treated or post-treated with CDDO-Me. Radioprotective effects of CDDO-Me were evaluated by assessment of γ-H2AX and 53BP1 foci by a fully automated microscopic system (TissueFaxs and StrataQuest). Double positive foci were considered as double-strand breaks. Expression levels of Nrf2-regulated proteins were assessed by western blot analysis. Cytokines were quantified using a multiplex immunoassay that allowed detection of 27 analytes.

Results: Post-treatment of NHEK with CDDO-Me prior to SM exposure resulted in a significant decrease of pro-inflammatory cytokines. DNA doublestrand breaks after radiation exposure were considerably reduced in the pretreated CDDO-Me therapy group most probably by preventing ROS-induced DNA damage due to induction of Nrf2-associated anti-oxidative proteins, e.g. hemoxygenase-1 (HO-1).

Conclusion: CDDO-Me was found to counteract cytotoxicity after epidermal SM and radiation exposure. Thus, a promising therapeutic compound addressing these different noxae was identified.

Effect of necrosulfonamide on sulfur mustard toxicity *in vitro*

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Introduction: Sulfur mustard (SM) is a vesicant agent which has been used in several conflicts of the 20th century. Most recently the OPCW officially confirmed an attack with SM in Syria 2015, which brought the difficulties of the therapy of SM intoxications back into the spotlight. Characteristic signs after dermal SM-exposure are necrosis, apoptosis and inflammation. To this day, no causal antidote against SM-intoxications exists and there is a need for improved therapeutic means.

Methods: To identify potential candidate substances for further research, a coculture of keratinocytes (HaCaT) and immunocompetent cells (THP-1) was established and exposed to SM-concentrations of 100, 200 and 300 µM. One hour post-exposure, a treatment with 1, 5 and 10 µM necrosulfonamide (NSA) was performed. Necrosis (ToxiLight), apoptosis (CDDE) and inflammation (interleukin 6 and 8 ELISA) were assessed.

Results: Treatment with NSA led to a highly significant reduction of apoptosis, which was decreased up to 80 % when compared to the sham-treated groups. Inflammation, which was assessed by interleukin-6-production, was also considerably mitigated (up to 50 %).

Conclusion: NSA is a highly effective substance to counteract SM-toxicity *in vitro*. Two major pathways of SM-pathophysiology (apoptosis and inflammation) were significantly affected. Therefore, NSA is most promising for further research, including *in vivo* studies.

Sulfur mustard resistant cells exhibit an improved response to oxidative stress

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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 Iraq last year. This emphasizes the importance to develop countermeasures against chemical warfare agents. Despite intensive research, there is still no antidote or prophylaxis available against SM.

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 and expression of GST, glutamate cysteine ligase (GCL) and glutathione-disulfide reductase (GSR) were investigated.

The HaCaT/SM cells showed not only the already described resistance against SM and various other alkylating agents, but also against hydrogen peroxide (H_2O_2). They exhibit more GSH even after SM treatment. Inhibition of GSTs led to sensitization to SM and a lower expression of GST was observed. The cells also expressed less GCL and GSR. The increased $NADP^+$/NADPH ratio might indicate that less NADPH is needed to reduce GSSG to GSH. The described deficiency of NAD^+ under acute SM exposure could not be confirmed under the chosen experimental conditions.

In summary, an improved response of the resistant cell line to oxidative stress was observed. The established cells clearly show a positive effect on the SM resistance through increased GSH levels, altered levels of reduction equivalents and GSH enzymes. Further research will show whether additional factors have an impact on the resistance.

Cytokines are able to reactivate the sulfur mustardinduced reduction of MSC migration

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After sulfur mustard (SM) exposure, the most prominent clinical symptom is the development of extensive non-healing skin wounds. This chronic wound healing dysfunction persists over long time. Mesenchymal stem cells (MSC) are known to play an important role in wound healing. Moreover, it is also known that patients with chronic wound healing diseases do have compromised mesenchymal stem cell functionality. Based on these observations and the known relationship between wound healing dysfunction and MSC function we investigated the impact of SM on human MSC.

MSC were isolated from femoral head of healthy donors. After MSC exposure with different SM concentrations we analysed cell survival, cell aging, migration ability and capacity of tissue specific differentiation.

MSC demonstrated an unexpected high tolerance against toxic concentrations of SM. Interestingly, the differentiation capacity was not significantly affected. On the other hand a SM exposure showed negative effects on the migration capability. The cells demonstrated an accelerated senescence. The reduced migratory capacity can be compensated in part by the addition of cytokines.

The effect of SM on MSC might play an important role in the persistence of long-term adverse effects, here could particularly the reduced migration be important. The compensation of the SM-induced migration reduction by addition of cytokines could be an option to resolve this problem. Moreover, our current results will help to understand the relationship between alkylating agents and MSC and thus will also give guidance in the future perspective for the therapeutic use of MSC in patients suffering from SM poisoning.

Sulfur mustard resistance in keratinocytes results in miRNA expression alterations

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Numerous microRNAs (miRNAs) have been identified to be responsible for the resistance of tumor cells to cytostatics. Possibly, the same miRNAs also play a role in the sulfur mustard (SM)-resistance of the keratinocyte cell line HaCaT/ SM recently developed by us.

To investigate this hypothesis, the expression levels of 1920 miRNAs in total were analyzed and the differences of each miRNA calculated for HaCaT/SM in comparison to HaCaT controls. Furthermore, the effect of antagomirs on expression of the in HaCaT/SM upregulated miR-125b-2 - 3p and of mimic on the expression of the in HaCaT/SM downregulated miR-181b as well as their effect on cell survival was investigated.

Out of 1920 miRNAs analyzed, 49 were significantly up- and 29 were significantly downregulated in HaCaT/SM when compared to HaCaT controls. The overexpressed miR-125b-2 and the downregulated miR-181b are of special interest due to their involvement in migration, proliferation, apoptosis and resistance against chemotherapeutic agents. It was shown that specific counterregulation of these two miRNAs via mimics, respectively antagomirs, resulted in unchanged cell survival after SM treatment in both cell lines.

In summary, the extensive differences in miRNA expression patterns between these cell lines indicate that specific miRNAs may play a role in the resistance mechanism against SM. The miR-125b-2 and miR-181b alone are not responsible for the resistance development against SM. Improving the resistance in normal keratinocytes by treatment with either both miRNAs together or a different combination might be used as an initial step in drug development against SM.

Differences in DNA damage pattern between sulfur mustard resistant cell line HaCaT/SM and HaCaT cell line

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Sulfur mustard (SM) is a highly reactive chemical agent. A pivotal effect of SMintoxication is DNA damage, induced by mo-

no-and bifunctional alkylation, which results in impaired DNA replication. The developed SM-resistant cell line HaCaT/SM showed several molecular differences compared to its original SM-sensitive HaCaT cell line, including less SM-induced DNA-adducts and a smaller nuclei perimeter in HaCaT/SM. The aim of this study was to examine if SM-resistance in HaCaT/SM is connected to altered DNA damage pattern. Comet assays were performed to determine whether there are differences in DNA stability between HaCaT and HaCaT/SM. In order to compare DNA damage after SM-exposure in both cell lines, phosphorylated H2AX (γ-H2AX) was used as a marker for DNA-double-strand breaks. Furthermore DNA content of HaCaT/SM and HaCaT was quantified.

A reduced DNA content of HaCaT/SM compared to HaCaT was observed. DNA stability measured by comet assay showed a smaller comet tail length for HaCaT/SM compared to HaCaT, suggesting an enhanced ability to cope DNA damage. This assumption is in line with our results from experiments using γ-H2AX as a marker for DNA damage. After SM-exposure we observed a lower level of DNA-double-strand breaks in HaCaT/SM compared with HaCaT via γ-H2AX staining and in In-Cell Western blots.

In conclusion, we examined that there is a correlation between SM-induced DNA damage and acquired resistance of HaCaT/SM to SM. The smaller nuclei perimeter, observed in previous studies and the lower DNA content suggest a higher chromatin density in HaCaT/SM, which is known from resistant tumor cell lines. Further studies will be necessary to evaluate the effect of SM onto comet tail formation and to reveal DNA repair mechanisms in both cell lines.

Sulfur mustard resistant cell line HaCaT/SM is lacking TRPV4 channel expression

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Almost 100 years after the first use of sulfur mustard (SM) during World War I, there is still no antidote available. The first SM-resistant keratinocyte cell line HaCaT/SM, derived from the HaCaT cell line, provides a promising model for finding novel therapeutic approaches. There are various differences on cellular and molecular level between both cell lines, including size, clonogenicity and glutathione content. The aim of the present study was to further reveal how HaCaT/SM acquired resistance to SM, focusing on transient receptor potential (TRP) channels.

To assess whether there are differences in the TRP channel expression profile between HaCaT and HaCaT/SM, a PCR screening was performed. In order to examine effects of activation and inhibition of TRPV4 channel on SMresistance of the cells, we used different selective agonists (GSK-1016790A, 4α-PDD) and antagonists (HC-067047, RN-1734). The sensitivity of both cell lines to SM was determined with a cell viability assay (XTT).

Using PCR screening, differences in TRP channel expression profile were detected between HaCaT and HaCaT/SM. Lacking expression of TRPV4 in HaCaT/SM indicates a possible factor contributing to the acquired resistance. We could show that inhibition of TRPV4 channel has no effect on cell-viability after exposure to SM. More critical findings are those with TRPV4 agonists, activating the channel. Results demonstrate that TRPV4 activation leads to significant SM-sensitivity, because HaCaT/SM almost lost their resistance to SM.

In summary, our results highlight the importance of TRPV4 channel for the acquired resistance of HaCaT/SM to SM. Further investigations e.g. measuring Ca^{2+} levels in both cell lines will show if there are additional differences.

Characterization of the N-acetylcysteine scavenging potential against sulfur mustard using mass spectrometry

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The vesicant sulfur mustard (SM) is a banned chemical warfare agent for which no causal therapy exists. Therefore, research on antidotes and scavengers against SM is of high interest. The toxicity of SM is discussed to be due to alkylation of proteins and DNA. Consequently, substances with a scavenging effect should inhibit such alkylation processes. One potential scavenger is N-acetylcysteine (NAC). NAC has been shown to be beneficial when applied as a drug against SM poisoning *in vitro* and *in vivo*. However, it is unclear, whether beneficial effects are only due to physiological processes or also due to chemical scavenging. Therefore, aim of this study was to investigate the chemical scavenging effect of NAC under physiological conditions using high resolution mass spectrometry. As an indicator of alkylation of biomolecules human serum albumin (HSA), which is a well known biomarker for SM exposure, was applied. Stable adducts are formed between its Cys34 residue containing a free thiol-group and SM and NAC. In our group we have developed novel analytical methods to detect such HSA SM adducts. Even though, SM alkylated a portion of NAC under physiological conditions, HSA SM adducts were not reduced independent of applied NAC concentrations. Furthermore, kinetic measurements exhibited that NAC did not accelerate degradation velocity of SM when compared to hydrolysis in phosphate buffered saline. Accordingly, we conclude that chemical scavenging of SM under NAC therapy has minor influence and beneficial effects observed might rather be due to physiological processes. Nevertheless, this analytical procedure can easily be transferred to test other therapeutics for their scavenging potential against SM *in vitro*.

Effect of N-acetylcysteine and glutathione on alkylating agents-induced TRPA1-channel activation

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The Transient Receptor Potential A1 ion channels (TRPA1) were found to sense temperature, pain and to act as chemosensors for a plethora of noxious substances. TRPA1 activation results in a distinct increase of intracellular calcium (Ca^{2+}), which may then affect Ca^{2+}-dependent signaling-pathways. Earlier studies reported a direct link between TRPA1 and in-

flammation or histamine-independent itch – two symptoms that are also present after exposure to the chemical warfare agent sulfur mustard (SM). Our results demonstrated that alkylating agents (SM and 2-chloroethylethylsulfide (CEES)) activate TRPA1 in a dose-dependent manner with a direct influence on cytoxicity. This was counteracted by the use of specific TRPA1-blockers. Furthermore, we investigated the influence of the thiol compounds N-acetylcysteine (NAC) and glutathione (GSH) on SM-induced TRPA1 activation. While GSH had no effect on channel activity, NAC prevented TRPA1 activation by the specific agonist Allylisothiocyanate and SM. Mass spectrometry analysis clearly revealed that intracellular NAC- or GSH-levels remained unchanged in our experiments, thus suggesting effects of NAC at extracellular TRPA1 protein residues. In 2015 the crystal structure of TRPA1 was released, therby identifying possible bindingsites of electrophilic compounds. Cysteine or lysine residues in the N-terminal ankyrin repeat motives are supposed to undergo covalent modifications by reactive compounds with subsequent channel activation. However, whether this is the only mechanism of TRPA1 activation is discussed controversially. Our results also affirm the hypothesis that additional, probably extracellular moieties, are involved in TRPA1 channel regulation. The identification of relevant binding-sites will help to understand TRPA1 function in more detail and may thus allow the development of new therapies for TRPA1-mediated pathologies.

Oxidative stress is a key player in the pathogenesis induced by sulfur mustard following cutaneous exposure

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Dermal exposure to the vesicating agent, sulfur mustard (HD), leads to skin inflammation that is accompanied by additional cytotoxic processes and severe injury. The healing process of these lesions is relatively long and incomplete. In an attempt to find therapeutic treatments for dermal HD-induced burns, the role of oxidative stress was investigated.

Mice ears were exposed to liquid HD. Systemic treatment with N-Acetylcysteine (NAC) concomitant with or without topical treatment of the nonsteroidal antiinflammatory drug (NSAID), diclofenac, and the steroid, betamethasone, was administered once a day for 3 days. Skin biopsies were collected at various time points up to seven days post-exposure for biochemical and histological evaluation.

A dose dependent inflammation and edema were developed in the skin starting 6 hrs post-exposure. This was accompanied by increase in the lipid peroxidation marker malondialdenyde (MDA) and in ferric reduction ability of the tissue (FRAP) indicating the development of oxidative stress following HD exposure. Biochemical analyses of skin samples revealed that NAC had no effect on oxidative stress markers however the anti-inflammatory (AI) treatment, as expected, reduced both, edema and inflammation but also had some beneficial effect on the amount of MDA.

The results of this study substantiate the involvement of oxidative stress together with inflammation in the development of HD injury. Nonetheless, a treatment with the antioxidant NAC, had no beneficial effect and the AI treatment was effective but not sufficient to prevent the development of the pathological process. Further investigation is required to find a combination therapy that will include anti-inflammatory drugs together with antioxidants in order to effectively ameliorate oxidative stress, decrease acute injury and improve healing.

Mustards – from lethal weapons to the cancer chemotherapy

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Mustard agents, also known as sulfur mustard or mustard gas, were the most widely used weapons during World War. This compound was considered by IARC and described as carcinogenic to humans (Group 1). Several studies have consistently shown an increased risk for lung cancer among workers in mustard gas-production and among war veterans.

After war, it was realized that it also caused systemic effects such as leukopenia, dissolution of lymphoid tissue, aplasia of the bone marrow and ulceration of the gastrointestinal tract.

The delayed biological effects of low doses of these agents were found to be mainly against dividing cells causing, in particular, inhibition of cell division generally, abnormalities of mitotic morphology and chromosomal lesions. This suggested a possible role for this compound in cancer treatment, but after an exploratory study it was considered too toxic for systemic use.

A nitrogen analog of sulfur mustard known as mechlorethamine (mustine), was also initially conceived as a chemical weapon, but it was applied to a lymphosarcoma patient in 1943 following the observation in autopsies that exposure led to profound lymphoid and myeloid suppression after an air attack on a ship carrying a stock of this substance.

Nitrogen mustards are nonspecific DNA alkylating agents. Studies have shown decrease in tumors after treatment chlormethine (HN-2), which has become one of the most effective anticancer drugs. Mustine has been used for many years, but with time has been displaced by less toxic and more effective drugs.

However, doctors had found a new method of treating leukemia and lymphoma using chemicals to target cancer cells within the body. Today we know it under the generalized name of chemotherapy. One of the most terrible weapons ever known led, through research and innovation, to a medical treatment that has saved countless lives.

Targeting pro-angiogenic factors as a novel therapeutic strategy in reducing corneal neovascularization following ocular sulfur mustard exposure

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Purpose: To examine the ability of Aflibercept (fusion protein which serves as an anti-VEGF A, B and PIGF) in ameliorating corneal neovascularization (NV) following sulfur mustard (SM) exposure in the rabbit model and to compare its effects to Avastin.

Methods: Chemical sulfur mustard burn was induced in the right eye of NZW rabbits by exposure to SM vapor. Aflibercept (2 mg) was applied once to neovascularized eyes by subconjunctival injection at 4 weeks post exposure while Avastin (5 mg) was administered subconjunctival twice a week for three weeks. Non-treated exposed eyes served as a control.

A clinical follow-up was performed up to 12 weeks following exposure and digital photographs of the cornea were taken for measurement of blood vessels using the image analysis software. Eyes were taken to histology 2 and 4 weeks following treatment and evaluation of NV presence was determined by using H&E and Masson staining.

Results: Corneal NV developed, starting as early as two weeks after exposure, and was associated with the delayed development of limbal stem cell deficiency. A single treatment with Aflibercept at 4 weeks following exposure, significantly reduced the extent of NV already one week following injection, an effect which lasted for at least 8 weeks following treatment, while NV in the non-treated eyes continued to increase. The extensive reduction in corneal NV in the Aflibercept treated group was confirmed by histological evaluation. Avastin multiple treatment showed a benefit in NV reduction but to less extent.

Conclusions: A symptomatic treatment with Aflibercept presented a long-term benefit in corneal NV reduction following ocular sulfur mustard exposure. These findings show the robust anti-angiogenic efficacy of Aflibercept and demonstrated the advantage of Aflibercept over the other standard antiangiogenic therapy, Avastin, in ameliorating corneal NV.

Role of mast cells in mouse skin injury following sulfur mustard induced injury

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Sulfur mustard (SM) is a vesicating agent known to cause skin inflammation and blistering. Evidence suggests that inflammatory cells and mediators they generate are important in the pathogenic response to SM. In the present studies we investigated the role of mast cells in SM-induced skin injury using a murine vapor cup model of exposure. Mast cells, identified by toluidine blue staining, were localized in the dermis, adjacent to dermal appendages and at the dermal/ epidermal junction. In control mice, approximately 47 - 62 % of the mast cells were degranulated. Treatment of mice with SM (1.4 g/m^3 in air for 6 min) resulted in increased numbers of degranulated mast cells (82 - 90 %) at 1 - 14 days post-exposure. This was associated with increased expression of inflammatory markers including tumor necrosis factor-α, interleukin-1β, myeloperoxidase, cyclooxygenase-2 (COX-2) and inducible nitric oxide synthase. Treatment of mice with an indomethacin prodrug linked to an aromatic ester-carbonate that targets cyclooxygenases (COX) enzymes which generate proinflammatory eicosanoids, and 3,3-dimethyl-1-butanol, a choline bioisostere that inhibits acetylcholinesterase (1 % in an ointment, twice daily) was found to reduce mast cell degranulation from 90 % to 45 % 1 - 3 days post SM and from 80 % to 40 % 7 - 14 days post SM. This was associated with a decrease in SM-induced double stranded DNA damage, as indicated by a reduction in epidermal cell expression of phospho-H2AX, as well as expression of markers of inflammation including COX-2 and myeloperoxidase. Taken together, these data suggest that the bi-functional indomethacin prodrug reduces inflammation, at least in part, by reducing degranulation of mast cells. The use of inhibitors of mast cell degranulation may be an effective strategy for mitigating skin injury induced by SM.

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Long-term neurological, neuropsychological and psychiatric complications of sulfur mustard and Lewisite mixture poisoning in Chinese victims exposed to chemical warfare agents abandoned at the end of WWII

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Objective: To assess the long-term neurological, neuropsychological and psychiatric consequences following acute exposure to a mixture of sulfur mustard (SM) and Lewisite among victims in Qiqihar, China.

Background: In early August 2003, 44 victims were poisoned by chemical warfare agents (CWAs) leaked from five tanks dug out at a construction site in Qiqihar, Northeast China. The tanks contained a mixture of SM and Lewisite abandoned by Japanese troops during World War II. Victims are still suffering from the late complications of them. There is paucity of information about the late toxic effects of SM and Lewisite.

Design/Methods: From March 2010 to March 2014, we have carried out neurological examination, including autonomic nervous function tests – the Cold pressor test (CPT), Active standing test (AST), EKG CVRR, and neuropsychological batteries in 19 victims (17 male and 2 female). The victims also completed Beck Depression Inventry and 17 item PTSD questionare. *Results:* The mean age of the victims was 42.4±12.3 (20 - 66) at the time of the latest examination in 2014. Severe autonomic nervous dysfunction such as hyperhidrosis, pollakisuria, diarrhea, and diminished libido, appeared in almost all victims. Polyneuropathy 29 %, contricted vision 29 %, mild ataxia 8 %. The rates of abnormal response on CPT, AST, and CVRR was 52.6 %, 42.1 %, 15 %, respectively. On neuropsychological testing, short-term memories and visuospatial abilities were frequently affected (58 %). 42 % of the victims showed generalized cognitive decline. Finally we found very high prevalence rates for severe depressive symptoms (79 %) and PTSD (74 %).

Conclusions: Long-term complications of acute exposure to sulfur mustard and Lewisite mixture compound have frequent and significant adverse consequences in neurological, neuropsychological, and psychiatric function of the victims in Qiqihar, China.

Verification of sulfur mustard exposure: essential and reliable progress in plasma sample shipping and analysis based on the dried blood spot technique

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Sulfur mustard (SM) is a banned chemical warfare agent causing blisters on exposed skin areas. Even though SM is banned by the Chemical Weapons Convention numerous incidents with a huge number of victims were reported during the ongoing Syrian Arab Republic conflict.

For biomedical verification of SM-exposure we have established diverse methods to detect variants of human serum albumin (HSA) modified at its only free cysteine residue (Cys34) after covalent reaction with SM representing a reliable biomarker in plasma (adduct). Analysis requires initial separation of the plasma fraction from freshly drawn whole blood and subsequent shipment to specialized laboratories. Shipping of liquid or frozen plasma by air, railway or on the road is subject to strict guidelines for complex packaging and therefore a major problem of sample shipment by deployed troops. In contrast, dried blood and plasma do not underlie these restrictions. To pay attention to often limited infrastructure in war zones and crisis regions we developed and characterized original approaches for dried plasma sample storage, shipment and preparation making use of filter paper and novel most modern microsampling devices. Dried SM-exposed plasma was suspended in buffer and proteolyzed by pronase to detect the biomarker by µLC-MS/MS (micro liquid chromatographytandem-mass spectrometry).

Excellent selectivity, sensitivity, and linearity were found for all devices. Stability of the HSA-adduct in dried plasma was shown under conditions of 3 climatic zones (temperate climate, hot and dry climate, and hot and humid climate) over 9 days simulating delayed shipping.

These novel procedures will be of high practical relevance for the medical service and extend the methods repertoire used for verification of SM-poisoning.

Genomic and genotoxic response characteristics of the sulfur mustard resistant cell line HaCaT/SM

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The skin represents the first line defense against environmental stressors and toxins. Recently, prolonged exposure to sulfur mustard (SM) was used to generate the SM-resistant HaCaT keratinocyte subline HaCaT/SM, displaying a 4.7-fold increased SM tolerance. So far, the causes for this behavior have remained elusive. To investigate the underlying mechanisms of the acquired SM resistance we studied the genomic and epigenomic characteristics that distinguish the sensitive and the SM-resistant subline.

First, we found that SM exposure reduced the average chromosome number from 76 in HaCaT to 55 in HaCaT/SM. The DNA damage response as measured by γ-H2AX foci showed that double-strand break induction by ionizing radiation was similar in the two lines, while DNA repair progressed faster in HaCaT/SM, indicating an altered response to genotoxic exposure.

Genomic analyses at basepair resolution by whole genome, paired-end deep sequencing and array CGH using genomewide microarrays identified a number of changes specific to HaCaT/SM, while shared patterns of point mutations and structural chromosome abnormalities underpin the common origin of both HaCaT sublines. Screening for differentially methylated DNA segments identified differentially methylated regions encompassing several 100 kb. Furthermore, we noted DNA copy number changes affecting genes involved in DNA repair and tumor suppression.

In all, it appears that the mechanisms causing the observed higher tolerance towards toxic stressors in the resistant HaCaT line relate to an altered defense against genotoxic exposures. Further analyses will identify genomic and epigenomic modifications associated with SM resistance.

A Syrian family who were exposed to blister agent

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Sulphur mustard (SM) was first used near Ypres, Belgium on July 12, 1917. After 100 years, today, we are still having no specific antidote for SM and for this reason it is still a dangerous enemy. On August 21, 2015, early in the morning, a non-state actor artillery position hit Marea city, Aleppo with shells containing blister agent and more than 50 civilians were affected from this chemical attack. One of those shell hit the roof of a family's home where parents, a 3-years old girl, and a 5-days old newborn girl were living. A garlicsmelling dark colored smoke filled the home. They suffered no early symptoms at the beginning but as hours passed they were falling ill. In the afternoon they were admitted to a NGO field hospital in nearby Tel Rifaat presenting conjunctivitis and respiratory difficulties. They were transferred to the other side of the border, to Kilis State Hospital, Turkey with an initial diagnosis of blister agent exposure. After the whole family were admitted to the emergency department at midnight, they were diagnosed as "second degree chemical burn". Medical decontamination was performed by hospital medical CBRN response team. Symptomatic treatment was provided. After a detailed medical examination, parents were urgently transferred to an intensive care unit (ICU) in Gaziantep, Turkey on August 22, 2015 because of widespread blisters and severe respiratory distress. Parents were discharged from ICU after five weeks. The older girl who presented itching, erythema and blisters after 48 hours was transferred to another hospital in Gaziantep on August 23, 2015 and she was discharged from the pediatric clinic at the end of first week. The newborn girl was transferred to a neonatal ICU in Gaziantep on August 24, 2015 and she died from severe respiratory failure and secondary bacterial infection due to bone marrow suppression on September 04, 2015.

Alternative treatment modality for healing of skin lesions in victims of blister agent

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Blisters are major skin symptom in patients who were exposed to vesicant agents. Blister forming could take 2 to 8 hours after a latent period which is followed by burning, itching, and erythema. 13 people were exposed to blister agent that was used as part of an improvised explosive device (IED) at Al Bab, Syria. They were transferred to the Turkey border after 18 hours from the chemical incident. Their initial medical decontamination was performed before they were transferred to Sehitkamil State Hospital, Gaziantep, Turkey. There, 13 patients were hospitalized in an isolated intensive care unit. Skin lesions including intact and ruptured blisters were observed mostly on upper extremities and anterior aspects of elbows. The mean burned body surface area was 18.3% (1%-45%) for 13 patients. 0.1% octenidine dihydrochloride solution (Oseptin®) and 0.2% polyhexanide gel (Cosmoburn®) were

administered to skin lesions of all patients twice daily and then lesions were left open to heal. No systemic antibiotic prophylaxis was preferred. Reepitalization was observed in all cases at the fifth day of the treatment and there was no secondary infection noted. At day thirty, wounds were almost completely healed. Routine debridement followed by topical paraffin, silver sulfadiazine, dexpanthenol, and flumethasone application were used for wound healing of sulfur mustard skin lesions in Iranian veterans between 1984 and 1985. In our cases we began to the treatment on their admission and we did not prefer prophylactic antibiotics. We concluded that antiseptic solution administration and wound irrigation with special gels speeded up the wound-healing process without secondary infections. It was also found that this treatment was more effective than debridement and systemic antibiotics as it increased patient's life quality by controlling pain, smell, and the condition of surrounding tissues.

Development of a mass spectrometric platform for the quantitation of mustard-induced nucleic acid damage

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The bi-functional DNA alkylating agent sulfur mustard (SM) was used as a chemical warfare agent. Although SM has been banned in most countries, its use in terroristic attacks or asymmetrical conflicts still represents a significant threat. The verification of SM-induced nucleic acid damage is mainly based on immunohistochemical methods, which have several limitations such as restricted specificity, sensitivity, and low dynamic range of quantitation. We have developed a UPLC-MS/MS-based platform for the quantitation of the most common mustard-induced DNA adducts, including bis(N7-guanineethyl) sulfide DNA crosslinks. We have established methods for the quantitation of the several common DNA adducts induced by SM. To this end, purification protocols, chromatographic conditions and mass spectrometric settings were developed to detect N7-hydroxyethylthioethyl-2´desoxyguanosine (N7HETE-dG) and N3-hydroxyethylthioethyl-2´desoxyadenosine (N3-HETE-dA) and their thermal hydrolysis products N7-hydroxyethylthioethyl-guanine (N7-HETE-Gua) and N3-hydroxyethylthioethyl-adenine (N3-HETE-Ade), respectively. Additional DNA adducts of the mono-functional SM derivative 2-chloroethyl ethyl sulfide ("half mustard", CEES) were analyzed. The stability of DNA adducts was investigated up to 6 days after damage induction and also compared to the stability of RNA adducts, as an alternative biomarker. In this project HaCaT and A549 cells were used, as they are derived from the two main targeted organs of SM intoxication. Additional non-radioactive isotope-labelled standards for isotope dilution MS approach were synthesized to account for technical variability during sample work-up and to improve MSbased quantitation. In conclusion, when fully established, this procedure should require a low amount of cellular material and could therefore be transferred to the quantitation of DNA adducts in human blood samples.

Identification of novel disulfide adducts between the thiol-containing leaving group of the nerve agent VX and cysteine-containing tripeptides derived from human serum albumin

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The ongoing Syrian Arab Republic conflict clearly demonstrates that chemical warfare agents still represent a considerable threat to military personnel and the civilian population. Such compounds are prohibited by the Chemical Weapons Convention and its violation will be of high political and legal impact. Therefore, reliable analytical methods for verification of an alleged use of banned substances are required. Apart from detection of the unaltered incorporated agents as well as their biotransformation products, current research is focusing on long-term biomarkers obtained from covalent adducts with biomolecules such as proteins.

Recently, we have introduced a microbore liquid chromatography-electrospray ionization high-resolution tandem-mass spectrometry (µLC-ESI MS/HR MS) method allowing detection of already known adducts between human serum albumin (HSA) and the nerve agent VX (phosphonylated tyrosine residues) and novel disulfide adducts at cysteine residues. Biomarkers were produced by enzymatic cleavage with pronase and detected simultaneously. Notably, the thiol containing leaving group of VX (2-(diisopropylamino)ethanethiol, DPAET) formed disulfide adducts that were released as cysteine and proline containing dipeptides originating from at least two different sites of HSA. Using synthetic peptide reference compounds two novel tripeptides were identified representing disulfide adducts with DPAET (Met-Pro-Cys-DPAET, MPCDPAET and Asp-Ile-Cys-DPAET, DIC-DPAET). Finally, the limit of detection for MPC-DPAET was evaluated, revealing toxicologically relevant VX plasma concentrations.

In conclusion, elucidation of the reactivity of VX with endogenous compounds allows identification of novel biomarkers appropriate for confirmation of an alleged use of prohibited CWA.

Functional measurements of nicotinic acetylcholine receptors – High-throughput screening of potential antidotes for the treatment of nerve agent poisoning

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Treatment of poisoning by organophosphorus compounds (OPC) is still insufficient due to the properties of various OPC and rapid aging of several OPCacetylcholinesterase (AChE)-complexes. Inhibited AChE results in accumulation of acetylcholine in the synaptic cleft and thus the desensitization of nicotinic acetylcholine receptors (nAChR) in the postsynaptic membrane is provoked. Direct targeting of nAChRs to recover receptors from desensitization might be an alternative therapeutic approach. In addition to the affinity of ligands towards the nAChRs, their functional properties are topics of interest. Bilayer methods (socalled "cell-free electrophysiology") based on solid supported membranes (SSM) are useful for activity measurements of electrogenic membrane proteins (e.g. ligand-gated ion channels like nAChRs) which are not accessible for patch clamp. Using native membrane fractions of *Torpedo californica electroplax*, functional measurements include different setups. By rapid exchange of non-activating, activating and desensitizing buffer, shifts into the conformations of resting, activated and desensitized states are forced. Especially, the interaction of ligands with the desensitized receptor state and the potency to recover from desensitization are important to detect their properties of positive allosteric modulators (PAMs). The recently developed method was transferred to the fully parallel 96 well-based platform SURFE2R 96SE (Nanion Technologies, Munich), the first instrument on the market featuring the SSM-technology in a high-throughput manner. This upscaling allows repetitive measurements of test batteries including positive and negative controls as well as multiple ligand concentrations within one single run. The results demonstrate that SSM-based electrophysiology is well suited for the detailed functional screening of nAChR-active compounds and has, because of its robustness and scalability, great potential for drug discovery.

Synthesis of new modulators of the nAChR for the therapy of poisoning by organophosphorus compounds

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For several nerve agents, the efficacy of the atropine and oxime standard therapy for poisoning by organophosphorus compounds is limited. Whereas atropine reduces overstimulation of the muscarinic receptors, insufficient oxime-induced reactivation of inhibited acetylcholinesterase finally leads to accumulation of acetylcholine in the synaptic cleft. Desensitization of the nicotinic acetylcholine receptors (nAChR) and inhibition of the cholinergic neurotransmission are the deadly consequences. Therefore, alternative therapeutic strategies counteracting the nAChR desensitization are strongly required to close this therapeutic gap.

Previous studies identified 4-*tert*-butyl substituted bispyridinium salt MB327 as a promising compound, able to restore soman-blocked neuromuscular transmission and sufficient to protect nerve agent poisoned guinea pigs when used together with physostigmine and hyoscine. Furthermore, MB327 has recently been found to act as a type II positive allosteric modulator of the nAChR. To get a deeper insight into the effects of bispyridinium non-oximes on the nAChR and for the development of structure-activity relationships with the aim of finding more potent resensitizing modulators, MB327 was chosen as lead structure. Supported by molecular modeling studies and guided by pharmacological test results, a variety of new bispyridinium salts has been synthesized. In this context new synthetic strategies were developed, providing an efficient and flexible access to differently substituted bispyridinium compounds.

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Electrophysiological assessment of bispyridinium compounds for their ability to recover human desensitized α7 nicotinic acetylcholine receptors

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The primary toxic action of organophosphorus (OP) compounds is the irreversible inhibition of acetylcholinesterase (AChE), impairing hydrolysis of acetylcholine (ACh). Accumulation of ACh within cholinergic synapses gives rise to overstimulation of nicotinic (nAChR) and muscarinic (mAChR) receptors causing a cholinergic syndrome. Medical countermeasure of OP poisoning comprises the use of oximes as AChE reactivators and atropine as noncompetitive mAChR antagonist. Since the currently available pharmacotherapy mitigates only muscarinic and not nicotinic effects directly, the need for such a therapeutic strategy is urgent, especially in cases of nerve agents resistant to reactivation by oximes.

Accordingly, direct intervention at nAChR may be a promising generic approach against nerve agent intoxications as postulated for the bispyridinium (BP) compound MB327, and may further counter acute respiratory depression which arises from desensitization of nAChR. In this context, BP compounds structurally related to MB327 were screened for their ability to recover human α7 nAChR from desensitization in stably transfected CHO cells applying an automated Patch Clamp system. Desensitization was induced by applying an excess of nicotine (1 mM) to the receptor population revealing a normal activation at a concentration of 100 µM. Although less pronounced than MB327, BP compounds substituted with a tert-butyl or a methoxy group at position 2, 3 and 4 were able to recover desensitized nAChR and prolonged the mean channel open time. As such, those agents are expected to act as positive allosteric modulators.

In conclusion, this functional screening assay plays an important role in rational drug design since it serves to identify antidote candidates, able to restore nAChR function after OP intoxication.

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Development of MS binding assays targeting the binding site of bispyridinium compounds at the nicotinic acetylcholine receptor

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Organophosphate poisoning causes accumulation of the neurotransmitter acetylcholine due to a permanent block of acetylcholinesterase at the synaptic cleft, which shifts the nicotinic acetylcholine receptor (nAChR) into a desensitized state. Since the atropine and oxime standard therapy is limited, there is an urgent need to find new therapeutic strategies, such as a direct intervention at the nAChR. As previously discovered in electrophysiological experiments on the nAChR from the Pacific electric ray (*Torpedo californica*) the symmetrical bispyridinium compound MB327 interacts directly with the nAChR, is able to positively modulate agonist induced ion channel activation and even to counteract desensitization. These characteristics nominate MB327 to serve as a lead compound for the development of even more potent drug candidates.

To enable the targeted search for potent ligands with the therapeutic profile of MB327, MS Binding Assays were developed, which use a centrifugation step to separate bound from non-bound marker. The strategy of MS Binding Assays, recently established in our group, follows the concept of radioligand binding assays, however, in contrast to the latter, is based on a native, non-labelled marker which is typically quantified by LC-MS/MS.

To characterize binding of MB327 to the nAChR from *Torpedo californica* saturation experiments were conducted. In addition, factors potentially influencing binding have been studied. Despite a very high amount of nonspecific binding, both affinity and the number of binding sites could be determined based on the saturation isotherms generated for MB327.

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Molecular modeling of nicotinic acetylcholine receptor modulators

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Irreversible inhibition of the enzyme acetylcholinesterase by organophosphorus nerve agents leads to an accumulation of the neurotransmitter acetylcholine (ACh) in the synaptic cleft. This results in a cholinergic crisis by overstimulating the nicotinic acetylcholine receptors (nAChR) which is switching into a desensitized state blocking the cholinergic neurotransmission. Recent findings identified the symmetrical 4-tert-butyl substituted bispyridinium compound MB327 exhibiting a significant potential as a positive allosteric modulator type II (PAMII) of nAChR at least partially re-sensitizing the desensitized nAChR and restore the muscular activity. However, so far nothing is known about the possible binding sites of MB327 and other bispyridinium compounds with similar potential or the mode of action required for the re-sensitization of nAChR.

For the identification of potentially new PAMIIs for nAChR we have conducted ligand-based pharmacophore design based on previous results. In order to enable the use of structure-based drug design methods we virtually screened the accessible surface of the nAChR of *torpedo marmorata* to identify potential binding sites of MB327 by means of molecular docking techniques. The results indicate that at least two potential binding sites for MB327 at nAChR exist in the channel pore in which MB327 intercalates between certain subunits of nAChR. In addition, inspection of these sites reveal that the unsymmetrical substitution of bispyridinium compounds should have a favorable effect on the pharmacological function.

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Investigations of albumin adducts as potential biomarkers of organophosphorus pesticide intoxication

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A systematic analytical verification method was developed to prove organophosphorus pesticide poisoning focusing on albumin adducts.

Human serum albumin (HSA) and serum were incubated with the pesticides paraoxon-ethyl and parathion-ethyl *in vitro* and treated with the proteases pronase, pepsin and trypsin separately. Resulting peptides and amino acids containing the phosphorylated tyrosine residues 411 and 138 were detected via microbore liquid chromatography-electrospray ionization high-resolution tandem-mass spectrometry (µLC-ESI MS/HR MS). Method optimization included e.g. elaboration of MS/MS-parameters, concentration of pepsin and trypsin and time of proteolysis. The limit of detection (LOD) was determined and the method was applied to *in vitro* samples using other pesticides and to real samples of a pesticide-poisoned patient.

Treatment with pronase produced a single phosphorylated tyrosine residue. Cleavage by pepsin resulted in the albumin-derived peptides LVRY^{*411}TKKVPQVSTPTL and VRY^{*411}TKKVPQVSTPT. Proteolysis with trypsin yielded the peptides Y^{*411}TK and Y^{*138}LYEIAR.

The LOD varied between an incubation concentration of 0.25 µM paraoxonethyl (phosphorylated tyrosine) and 64.0 µM (peptide Y^{*138}LYEIAR). The yield of the phosphorylated products showed intra- and interspecies variability. Differences were also found after incubation of serum and HSA with different pesticides indicating differing reactivities with the tyrosine residues 411 and 138. Plasma samples of a patient, who was poisoned with pirimiphos-methyl, revealed a 37 h half-life of the corresponding HSA-adduct.

Important parameters and considerations for *in vitro* reactivation assays of OP-inhibited cholinesterase

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The risk of organophosphate (OP) poisoning is still present, currently mostly as occupational hazard in agriculture (i.e. pesticides), but there is also justified concern about the potential abuse of OP compounds as nerve agents. Standard oxime reactivators (2-PAM, HI-6, TMB-4, obidoxime) lack antidotal effi-

ciency and/or the optimal pharmacological characteristics; therefore, new reactivators of OP-inhibited acetylcholinesterase are being synthesized. In order to evaluate their efficiency as reactivators, many research groups are employing reactivation tests based on the spectrometric Ellman assay. Although the method seems simple, the determination of reactivator efficiency and evaluation of kinetic constants are mostly incomparable among laboratories. Therefore, we addressed the need to standardize the reactivation assay with an evaluation of important factors for experimental design, such as side-reactions between reactants: enzyme, oxime, the Ellman reagent, and substrate. Additionally, concentrations of all of the reactants should be taken into consideration and presented in detail, e.g. there has been significant difference in the results depending on the concentration of the inhibited enzyme in the reactivation mixture, which has not been emphasized until now. Finally, the presented results can differ significantly depending on the evaluation of reactivation constants and overall data analysis. This study was meant to emphasize the important steps in the determination of reactivation parameters and suggest a uniform experimental design, data analysis and result presentation with an aim to achieve better agreement and comparability between the results of different laboratories, and overall a more efficient evaluation of *in vitro* reactivation potency.

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New uncharged potent reactivators of AChE and BChE inhibited by nerve agents

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Intoxication by organophosphorus nerve agents (OPNA) leads to the irreversible inhibition of enzymes acetylcholinesterase (AChE) and butyrylcholinesterase (BChE). This inhibition causes acetylcholine accumulation in the peripheral and central nervous system synapses, leading to a cholinergic crisis. The most prominent symptoms with a possible lethal outcome are respiratory failure and seizures, but those who survive can also experience long-term neurological impairments, e.g. cognitive and behavioural incapacitations. The antidote treatment comprises an antimuscarinic drug, an oxime reactivator and an anticonvulsive drug. Standard reactivators (2-PAM, HI-6, LüH-6, TMB-4) are not efficient for every OPNA, and since they have positively charged quaternary nitrogen in their structures, they cross the brain-blood barrier poorly. We synthesised and evaluated novel uncharged and therefore possibly centrally active reactivators. They showed a highly promising *in vitro* reactivation profile for VX-inhibited AChE/BChE, sarin-inhibited AChE and cyclosarin-inhibited BChE. Using molecular docking, the possible interactions that these oximes form with the amino acid residues in the active site gorge of the enzymes near the covalently linked OPNA, were detected. Additionally, *in silico* determined physicochemical properties (e.g. lipophilicity, polar surface area, hydrogen bond donors and acceptors, pKa) implied that these novel oximes were likely to be centrally active. On the other side, the higher lipophilicity was connected with a higher probability of accumulation in different tissues and therefore could have led to adverse effects. Further evaluation of these novel reactivators' *in vivo* efficacy is needed, as well as evaluations of their pharmacokinetic and toxicological profiles.

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Advances in the treatment of the ocular insult, miosis and visual dysfunction, following ocular exposure to the nerve agent sarin

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Purpose: Eye exposure to the organophosphorus irreversible acetylcholinesterase inhibitor sarin results in long-term miosis (a reduction of at least 50 % of pupil width) and reduction in visual function. Anti-cholinergic drugs, such as atropine, are used topically in order to counter these effects and obtain symptomatic relief. Unfortunately, such compounds attenuate ocular discomfort at the expense of producing mydriasis and partial cycloplegia symptoms, which may worsen visual performance. This study was aimed to test the beneficial effect of short-acting anti-cholinergic drugs combined with oximes in contradicting the sarin-induced miosis and visual impairments. Methods: Male Pigmented Long-Evans rats were topically exposed to sarin (0 - 10 µg). Dose response relationship of pupil width was evaluated by an infrared-capable video camera 15 min-72 h following the exposure and up to 8 h following anti-cholinergic and oxime treatments. Visual function assessment was performed using the "Cued" Morris Water Maze task following sarin exposure and following exposure and treatment.

Results: Rats showed a dose-dependent miosis, which returned to pre-exposure levels within 24 - 48 h. Significant reduction in visual function was seen in animals exposed to 0.2 µg sarin and above, 15 - 35 min following sarin exposure. Short-acting anti-cholinergic or oxime treatments differentially reduced the sarin-induced miosis and the resulting impairment in visual performance. Moreover, the combined treatment presented a rapid beneficial effect on the parameters evaluated.

Conclusions: The use of the topical combined short-acting anti-cholinergic treatment, tropicamide, with oximes following ocular sarin exposure rapidly widened pupils and improved the visual insult.

The evaluation of benefit of newly prepared reversible inhibitors of acetylcholinesterase and commonly used pyridostigmine as pharmacological pretreatment of somanpoisoned mice

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The ability of four newly prepared reversible inhibitors of acetylcholinesterase (6-chlorotacrine, 7-phenoxytacrine, compounds 1 and 2) and currently used carbamate pyridostigmine to increase the resistance of mice against soman and the efficacy of antidotal treatment of soman-poisoned mice was evaluated. The evaluation of the effect of pharmacological pretreatment is based on the identification of changes of soman-induced toxicity that was evaluated by the assessment of its LD50 value

and its 95 % confidence limit using probitlogarithmical analysis of death occurring within 24 h after administration of soman at five different doses with eight animals per dose. Among reversible inhibitors of acetylcholinesterase studied, only 6-chlorotacrine was able to markedly protect mice against acute toxicity of soman. In addition, the pharmacological pretreatment with 6-chlorotacrine was able to increase the efficacy of antidotal treatment (the oxime HI-6 in combination with atropine) of soman-poisoned mice. Another reversible inhibitor of acetylcholinesterase (compound 2) did not protect mice from acute toxicity of soman, but the pharmacological pretreatment with this compound was able to increase the efficacy of antidotal treatment of soman-poisoned mice. The other newly prepared reversible inhibitors of acetylcholinesterase (7-phenoxytacrine, compound 1) as well as commonly used pyridostigmine did not influence the efficacy of antidotal treatment. These findings demonstrate that pharmacological pretreatment of soman-poisoned mice can be promising and useful in the case of administration of 6-chlorotacrine and partly compound 2.

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Retigabine alleviates neurological damage following sarin exposure

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Background: Sarin is an irreversible organophosphate (OP) cholinesterase (ChE) inhibitor and a highly toxic, volatile warfare agent. Following the overt, dose-dependent toxic signs (e.g. tremor, hypersecretion, seizures, respiratory depression and eventually death), a widespread brain damage is often reported. This long-term brain damage which is correlated with seizure activity is mostly irreversible and may even deteriorate with time. Thus, many efforts are invested in searching for new therapeutic strategies for neuroprotection. The aim of the present study was to test the effect of retigabine (KCNQ voltage-gated potassium channel opener) on survival and neuroprotection following sarin exposure.

Method: Male Sprauge-Dawley rats were exposed to 1 x LD50 sarin (90 µg/kg, i.m.). Rats were treated upon toxic signs with retigabine (5 mg/kg, i.p., once). Brains were removed 24 h and 1 week later and processed for biochemical analysis and immunohistopathological examination.

Results: Immediate treatment with retigabine protected against sarin mortality (40 % mortality in non-treated vs. 8 % in treated rats). Furthermore, retigabine minimized or prevented all together seizure activity following sarin exposure. Retigabine lead to a significant reduction in gliosis, astrocytosis and neuronal damage as well as in the neuro-inflammatory markers IL-1beta and IL-6 levels. *Conclusions:* Retigabine is an effective antidote against OP poisoning even when administered alone. Retigabine may possibly be applied following organophosphate intoxication to alleviate neurological damage after onset of seizures and the initiation of secondary processes.

HI-6 assisted detoxification of VX and soman by a human AChE mutant in whole human blood

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In the event of nerve agent poisoning immediate medical intervention is vital due to the deadly effects caused by acetylcholinesterase (AChE, EC 3.1.1.7) inhibition. The outcome is especially adverse in case of soman, owing to the very rapid dealkylation (ageing) of the soman-AChE conjugate. Nowadays, exogenously administered human enzymes like butyrylcholinesterase or AChE mutants are investigated as supplemental bioscavengers with the intention of developing prophylaxis for first responder use. Our *in vitro* studies have shown that HI-6 is an efficient reactivator of the human AChE mutant Y337A/F338A upon VX and soman inhibition and that the soman-Y337A/F338A conjugate has a 50 min half time of aging (vs. 2 min for wild-type human AChE). Along these lines, we tested the bioscavenging potential of the Y337A/F338A AChE mutant when combined with HI-6 for samples of whole human blood treated with VX or soman. Our findings indeed indicated that blood cholinesterase activity was completely restored by this catalytic oxime-assisted scavenging system within 10 min in case of exposure to a 10-fold VX excess (with regard to the mutant) and treatment with 1 mM HI-6. A similar bioscavenging action was also noticeable in case of soman exposure. Therefore, we have shown that VX and soman detoxification is possible and effective in whole human blood by turnover cycles of Y337A/F338A inhibition and reactivation by HI-6. This combination scavenging system affords a potentially significant improvement in the treatment of VX and soman exposure. Moreover, further *in vivo* experiments on mice supported the *ex vivo* results showing that catalytic VX and soman scavenging improved therapeutic outcomes by delaying the onset of toxicity symptoms and preventing lethality.

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Experimental design considerations for the assessment of nerve agent medical countermeasures

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Efficacy studies of medical countermeasures (MedCM) for nerve agent poisoning have historically used 24 h as an experimental end-point. The pharmacological components of MedCM generally have short elimination half-times, such that therapeutic levels of these drugs are maintained for much less than 24 h. Based on likely casualty processing times, first aid MedCM would only be required to confer protection for 4 - 6 h, after which further medical support would be available. Therefore, it is unrealistic to assess MedCM (in isolation) beyond a time at which further treatment would likely be available and there is a danger that potentially effective MedCM could be rejected.

In a guinea-pig model of acute nerve agent poisoning, we assessed the effectiveness of first aid MedCM over 6 h. Studies were carried out to compare the oxime HI-6 and the antinicotinic MB327, in combination with atropine and avizafone, against

soman poisoning. A protection ratio design was used, and the results at 6 h were compared to previous 24 h data. As expected, at 6 h the protection ratio of the therapies against soman was increased; a modest improvement was seen for HI-6, but therapy including MB327 resulted in a substantial improvement in survival. Although MB327-treated animals survived to 6 h at high challenge doses of soman they were substantially incapacitated and further intervention would be necessary for longer-term survival. Future work will assess the integration of first aid therapy with supportive treatments, using experimental designs that reflect the capabilities available at the various levels of medical care.

Ability of aerosolized rHuBChE to protect against inhaled organophosphates

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Recent sarin use in Syria, pesticide use by terrorist in Afghanistan and the > 5 billion lbs of pesticides for agricultural purposes used annually highlight the urgent need for an antidote to protect against organophosphate (OP) toxicity. Although the efficacy of plasma-derived BChE prophylaxis in animals against multiple LD50s of nerve agents is well established, limited availability and high cost of the large doses required for protection, have led to a focus on a rHuBChE (recombinant human butyrylcholinesterase) countermeasure. However, delivery of rHuBChE by parenteral (i.v., i.m., s.c.) injection results in less than optimal pharmacokinetics. PlantVax has thus taken an alternate approach by developing an aerosolized (aer) aer-rHuBChE pretreatment to prevent neurotoxicity associated with inhaled nerve agent and pesticide (OP) exposure. This takes advantage of the fact that aer-rBChE molecules are too large to exit the lung and instead form a "pulmomary bioshield" that can scavenge incoming (inhaled) OPs *in situ* preventing their entry into the systemic circulation and inhibition of cholinesterases in blood and brain and ensuing neuro- and respiratory toxicity. To date, protection by aer-rHuBChE delivered using a nebulizer has been demonstrated in macaques against 330 µg of inhaled paraoxon in macaques given four days after the pretreatment. In addition, a post-exposure i.m. treatment with the oxime RS194B was able to effect reversal of severe clinical symptoms and survival in macaques which received substoichiometric doses of aer-rHuBChE 24 h prior to exposure with 50 µg/kg sarin vapor.

Testing betaglucuronidase as a biomarker of acute neurotoxic organophosphorus compounds poisoning

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Introduction: Organophosphorus compounds constitute a class of chemicals including insecticides and chemical warfare agents. Rapid diagnosis requires the identification of sensitive biomarkers. Toxicological studies argues betaglucuronidase validation as a sensitive biomarker of the organophosphorus compounds poisoning.

Objective of the study: Highlighting betaglucuronidase as biomarker in the organophosphorus compounds intoxication.

Methods: The experimental studies were performed in accordance with the principles of bioethics contained in Directive 2010/63 / EU, on Wistar rats, kept in suitable microclimate, divided into five homogeneous study groups. Each group has been administered paraoxon in doses 0.5, 1, 1.5, 2 x LD50. Two hours after intoxication whole blood was collected in order to determining betaglucuronidase and acetylcolinesterase values. Statistical analysis of the results were performed by using ANOVA, t-test and Correl.

Results: Probability associated with acetylcholinesterase averages showed a significant difference between intoxicated, treated and untreated groups and control group. A paraoxon correlated increased averages were showed in case of betaglucuronidase. The correlation coefficient between betaglucuronidase averages and those of paraoxon was 0.9905.

Conclusions: Paraoxon irreversibly inhibits acetylcholinesterase. Administration of reactivating obidoxime determined acetylcholinesterase restore values. An average increase of betaglucuronidase linked to the rise of paraoxon was evidenced by the correlation coefficient. Based on experimental results can thus conclude that betaglucuronidase can be considered, alongside acetylcholinesterase, a sensitive biomarker in neurotoxic organophosporus compounds poisoning.

The efficacy of HI-6 DMS as a sustained infusion against percutaneous VX poisoning in the guinea pig

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The current self/buddy aid for nerve agent poisoning fielded by the U.K. military has an anti-muscarinic component (atropine), an anti-convulsant (avizafone) and an oxime component, currently pralidoxime methane sulfonate (P2S) in an auto-injector. If further treatment is required, for example following percutaneous exposure to a low volatility agent such as VX, P2S is available for intravenous infusion by medical personnel. There is a programme of work to replace P2S in the auto-injector with an alternative oxime, HI-6, which is also being developed as a powder for solution to replace intravenous P2S.

As part of the development of HI-6, this study evaluated the efficacy of a sustained infusion of HI-6 DMS (dimethanesulphonate) in the conscious guinea-pig against a percutaneous exposure of 1.53 mg/kg VX. HI-6 DMS (0.24 or 0.48 mg/kg/min infused over 24 h) in combination with atropine (0.07 mg/kg/min), significantly increased survival over atropine alone – all HI-6 treated animals survived to 48 h, compared to less than 6 h for atropine alone. Doubling the concentration of HI-6 significantly increased both steady state levels of HI-6 and the reactivation of acetylcholinesterase, although this had no additional effect on survival or any of the physiological signs of poisoning.

An efficient nanodrug for detoxification of nerve agent soman in brain

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Nerve agent (NA) soman is the most toxic artificially synthesized compound that can rapidly penetrate into the brain, irreversibly inhibit acetylcholinesterase (AChE) activity causing immediate death and quickly aged leading to no antidotes for reactivating. However, almost no reactivators drugs could penetrate the blood–brain barrier (BBB). Moreover, there are currently few brain-targeted nanodrugs that can treat acute chemical brain poisoning owing to the limited drug-releasing speed and drug loading rate. Therefore, we design a nanodrug against NA toxicity that has high BBB penetration and is capable of rapid drug release, which composed by transferrin-modified mesoporous silica nanoparticles (TF-MSNs) as nano-carrier and the known AChE reactivator HI-6 as detoxification component. This nanodrug rapidly penetrated the BBB in zebrafish and mice and restored cerebral AChE activity via the released HI6, preventing the brain damage caused by soman poisoning and increasing the survival rate in mice. Furthermore, there was no toxicity associated with the MSNs in mice or rats. These results demonstrate that TF-MSNs loaded with HI-6 represent the most effective antidote against NA poisoning by soman reported to date, and suggest that MSNs are a safe alternative to conventional drugs and an optimal nanocarrier for treating brain poisoning, which requires acute pulse cerebral administration.

Stability of Reactive Skin Decontamination Lotion (RSDL®) under the conditions of the European Training Mission (EUTM) in Mali

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Reactive Skin Decontamination Lotion (RSDL®) is used for decontamination of Chemical Warfare Agents after dermal exposure. The Bundeswehr handles it also in missions abroad. In this work we describe the examination of stability of RSDL® under the conditions of the European Training Mission (EUTM) in Mali, which is climatic zone IV according to WHO guideline.

Samples were taken from June 2014 to May 2015 in the area of Koulikoro. Stability was determined by analysis of the active ingredient diacetylmonooxime (DAM) and the postulated degradation product dimethylglyoxime (DMG) over a period of 12 months. Analytical data were complemented by the temperature profile of RSDL® during mission and calculation of the corresponding Mean Kinetic Temperature (MKT). Besides stress tests were made and the data were used for calculations according to the Arrhenius equation. Based on the temperature dependent rate constants, the expected amount of DAM and DMG was calculated for the conditions of use during mission. These data were compared to the measured data from samples exposed in mission. By this procedure an evaluation of the testing-concept was accomplished.

The decline of DAM followed first order kinetics, while formation of DMG could be described by zero order kinetics. The rate constants were distinctively temperature dependent. Calculated data from Arrhenius fitted well to those measured from samples of mission.

Based on an acceptable DAM-content of 90 % (specification) and a valid threshold value of 0.1 % (w/w) for the degradation product DMG, RSDL® proved to be stable for 12 months under the conditions of EUTM at an overall MKT of 31 °C. Besides our data show that monitoring of temperature in mission is a suitable surrogate parameter for a risk based quality assessment of pharmaceutical products in missions.

Use of RSDL® (Reactive Skin Decontamination Lotion Kit) for removal of radioactive isotopes from skin

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Countermeasure tools are carried by soldiers and responders for the immediate removal and or neutralization of chemical warfare agents (CWA) post exposure. One in particular is the RSDL Kit, which is indicated for the removal or neutralization of CWAs in 2 minutes from intact skin.

Discussed are the results from an *in vitro* pilot study and an *in vivo* preclinical study to evaluate RSDL efficiency in the removal of non-water soluble Strontium-85 (^{85}Sr) and water soluble Iodine-125 (^{125}I) particles after dermal exposure on skin.

The results of the pilot study determined that leaving the radioisotope on the pig skin longer than 3 hours before initiating RSDL decontamination resulted in minimal removal of ^{85}Sr and in particular ^{125}I. In contrast, initiating decontamination with RSDL after a 2 minute skin exposure to the radioisotopes improved efficacy and the remaining contamination on the skin was less than 10 % on average.

The second *in vivo* study determined radioactivity on the skin surface after decontamination with the RSDL sponge followed by a dry wipe to remove residual lotion, left a normalized average of 11.8 % to 19.5 % radioactivity on the skin site following a 2 minute exposure time or a normalized average of 39.8 % to 46.3 % percent radioactivity on the skin site following a 60 minute exposure time.

For immediate use in a radio-isotope skin exposure incident, use of RSDL proved a decrease dermal exposure from both ^{85}Sr and ^{125}I and that for decontamination, and may be useful especially in the absence of soap and water.

The CULTEX® Radial Flow System (RFS): an innovative 3R-conform future *in vitro* model for the assessment of acute lung toxicity

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Introduction: Assessment of acute lung toxicity originating from airborne particles has gained attention due to regulatory requirement, as e.g. in the REACH (Registration, Evaluation, Authorization and Restriction of Chemicals) legislation. So far,

animal studies were conducted to analyze pulmonary toxicological effects of chemicals. However, especially the EU-regulation REACH demands to implement the 3R concept of refinement, reduction and replacement of *in vivo* experiments. The CULTEX® RFS closely mimics the *in vivo* situation of the alveola and allows exposure of human lung epithelial cells to airborne particles at the air-liquid-interface (ALI) with subsequent assessment of toxicological properties, thereby potentially replacing animal experiments. Objective: Purpose of this project is the thorough validation of the CULTEX® RFS method as a replacement for animal experiments in acute lung toxicology research.

Materials & Methods: A549 human lung epithelial cells were exposed either to clean air (process control) or to adjusted particle concentrations (25, 50 and 100 µg/cm²) at the ALI. Cell viability was determined 24 h after exposure using the WST-1 assay. Experiments were conducted in 3 different laboratories in order to document transferability and stability of the procedure. Collected data are the basis for a prediction model of acute lung toxicity.

Results: The harmonization and refinement of the methodological procedure resulted in highly stable and reproducible results across laboratories. Current results confirmed findings of a former pre-validation study (BMBF 0315710, 2013), thereby underlining the robustness and accuracy of the method.

Conclusion: The current results highlight the CULTEX® RFS method as an innovative 3R-conform approach for analyzing acute toxicity of inhalable substances in future.

Precision cut lung slices as test system for candidate therapeutics in organophosphate poisoning

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Poisoning with organophosphorus compounds (OP), which include nerve agents and pesticides, presents a substantial threat. Numerous casualties follow the dissemination of OP in military conflicts or by terroristic actions, as seen 2013 in the Syrian sarin attack. Additionally, intake of pesticides in suicidal intention or by accident accounts for many civilian deaths annually.

After OP incorporation, inhibition of the enzyme acetylcholinesterase results in accumulation of the neurotransmitter acetylcholine (ACh). Subsequent lungrelated symptoms, e.g. bronchoconstriction and bronchial hypersecretion, can ultimately lead to death. As standard therapy, a combination of an oxime and atropine are applied, a regimen that lacks efficacy in several cases of OP poisoning. Recent research yielded probable therapeutic compounds that still need further evaluation, e.g. modulators of the nicotinic and possibly the muscarinic ACh-receptor. Thus, aim of this study was the development of a test system for such substances, based on employment of precision cut lung slices (PCLS). As main parameter, airway area changes following ACh stimulation were examined by video-based microscopy. In control groups, ACh induced a spontaneously reversible airway contraction, as indicated by a primary decrease of the airway area to about 28 ± 4 % (mean ± SEM) of the initial area, followed by a spontaneous increase to about 58 ± 6 % by the end of the experiment. In OP poisoned PCLS, the ACh-induced airway contraction was irreversible, as shown by a constant decrease of the airway area, e.g. to about 11 ± 4 % for VX. This effect was rapidly antagonized after addition of atropine, showing the reversal of airway contraction as a clear marker for a therapeutic effect. Hence, an appropriate system to investigate effects of candidate therapeutic compounds on lung tissue is available now.

Civilian exposure to halogen pulmonary irritants: a systematic review

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Introduction: Halogen pulmonary irritants (HPIs) are volatile liquids that directly damage the respiratory mucosa. Chlorine is readily available in large volumes as an industrial chemical and has a significant potential for accidental or deliberate release, as observed in the current Syrian conflict. The majority of the medical literature on the clinical features and management of HPIs are derived from military experience. We conducted an open-source systematic review to determine the clinical features; treatment and long-term sequelae of civilian HPI exposure.

Methods: A systematic review was conducted using the Preferred Reporting Items for Systematic Reviews and Meta-Analyses (PRISMA) methodology. Briefly, the Medline; Ovid and Google Scholar databases were searched from 1966 to January 2017. Papers were included if the abstracts were available in English; details of the source of exposure was available and a description of clinical features present. A database of relevant papers was compiled and descriptive statistics used to summarise the data.

Results: Sixteen papers describing 31 incidents involving exposure to HPIs were identified. Thirty incidents concerned chlorine and one bromine. A total of 1,438 individuals (720 male and 718 female) were affected. The average age was 23.6 years. The most common reported features were cough (29 %), dyspnoea (25 %), excessive sputum (12 %) and haemoptysis (2 %). Acute management included high-flow oxygen (98 %); nebulizers (10 %); steroids (10 %); antibiotics (3 %) and ventilation (2 %). Eleven deaths (0.8 %) were reported (10 male). Follow-up data was often limited but full recovery was generally reported.

Discussion: Civilian HPI exposures reported in the medical literature were associated with respiratory symptoms. Management was generally supportive and the prognosis good for the majority of cases.

Treatment of sulfur dioxide-induced lung injury in rats

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Background: Inhalation of sulfur dioxide (SO_2) primarily affects the lungs and exposure to very high concentrations can be immediately dangerous to life. The response after inhalation of SO_2 implicates that the early pulmonary response involves tissue injury, neutrophilic lung inflammation and airway hyperresponsiveness (AHR). In rats, pulmonary fibrosis is evident 14

days post exposure as indicated by analysis of collagen deposition in lung tissue and early treatment with a single dose of dexamethasone (DEX) significantly downmodulates the acute inflammatory response in airways. However, this treatment is not sufficient for complete protection against the pulmonary toxicity.

Objectives: To evaluate whether repeated treatment with DEX alone or in combination with the antioxidant N-acetyl-L-cysteine (NAC) and the antifibrotic substance, pirfenidone (PFD), administered 1 h, 5 h and 23 h after SO_2exposure (2200 ppm, 10 min) could counteract the inflammatory responses, AHR and lung fibrosis in female Sprague-Dawley rats.

Results: All treatment approaches significantly reduced the total leukocyte response but only combined DEX and NAC reduced the number of neutrophils in BAL. PFD reduced methacholine-induced AHR to almost control levels, in contrast to DEX and DEX/NAC which only provided partial protection against AHR. Only DEX treatment reduced the collagen formation in lung tissue.

Conclusion: All three treatment protocols reduced the acute SO_2-induced inflammatory response in airways with combined DEX and NAC treatment slightly more efficient than the other protocols. PFD appeared to be more efficient than DEX and DEX/NAC combination in reduction of AHR. Only DEX treatment was efficient in the reduction of long-term effects monitored 14 days post exposure. In the future, studies addressing combination of both anti-inflammatory and anti-fibrotic treatment is highly motivated.

8-Isoprostane is an early biomarker for oxidative stress in chlorine-induced acute lung injury

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Inhalation of chlorine (Cl_2) may cause acute lung injury characterized by pulmonary edema, pneumonitis, and hyperreactive airways. The aim of the study was to identify possible biomarkers for Cl_2-induced acute lung injury (ALI).

Mice were exposed to Cl_2 for 15 min using two protocols 1) dose-response (25 - 200 ppm) and 2) time-kinetics (2 h - 14 days post-exposure). Markers for inflammatory response, lung permeability, blood coagulation and oxidative stress were analyzed and the specificity of the biomarkers were evaluated in mouse models for lung injury induced by nitrogen mustard analogue melphalan, sulphur dioxide (SO_2) or ammonia (NH_3).

Exposure to 50 - 200 ppm Cl_2 caused a concentration-dependent inflammatory response as indicated by increased level of IL-1, IL-6 and KC in bronchoalveolar lavage (BAL) 2 - 6 h after exposure which was followed by increased lung permeability and a prominent neutrophilic inflammation 12 - 24 h post exposure. The early cytokine response in BAL was associated with a clear but transient increase of 8-isoprostane with its maximum at 2 h after exposure. 8-isoprostane is considered as a biomarker for oxidative stress. A significant increase of 8-isoprostane could also be detected in serum 2 h after exposure to 200 ppm Cl_2. This was followed by a systemic inflammatory response as indicated by increased levels of IL-6 and KC and signs of increased fibrinogen and PAI-1 (role in blood coagulation and wound healing) in serum. Neither melphalan, SO_2 nor NH_3 exposure increased the 8-isoprostane levels, indicating that 8-isoprostane is induced in airways through direct oxidation by Cl_2. In a rat model of Cl_2-induced ALI, it was demonstrated that 8-isoprostane can be detected in exhaled breath condensate 24 h after exposure. We conclude that 8-isoprostane represents an early biomarker for oxidative stress in airways following inhalation exposure to Cl_2.

Atropine for rescue against mortality from acute high level chlorine inhalation

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Rationale: Chlorine (Cl_2) is a highly reactive oxidant gas that can be chemically weaponized. We have found that exposure to high levels of Cl_2 in rats causes severe hypoxemia, acidosis, poor cardiac output (CO), respiratory distress, hypotonia, seizures and death. We also found diminished cholinesterase (ChE) activity. To determine if the ChE pathway plays a key role in morbidity and mortality after Cl_2, we examined atropine therapy as rescue after high level Cl_2 exposure in rats.

Methods: Male Sprague-Dawley rats were exposed to Cl_2 gas (LD56 - 67) in a whole body awake exposure system, and observed via various clinical observation metrics for 6 h. One dose of atropine (10 mg/kg, i.m.) given ~7 - 10 minutes after exposure, was compared to 2 doses (2^{nd} dose at 2 h). A separate group of animals had intracardiac catheters placed for hemodynamics monitoring, and blood samples were collected for arterial blood gas analysis.

Results: Atropine treatment improved survival, best with 2 doses (82 % vs 42 % after Cl^2). Hypoxemia and bradypnea were not significantly improved. However, clinical distress (respiratory distress, activity) and neuromuscular scores (tone, posture, coordiantion, movement) both improved with atropine. Atropine administration improved blood acidosis (pH 7.40 vs 7.15 without treatment) seen after Cl_2 . Cardiac output was severely diminished after chlorine inhalation (from 140 to 80 ml/min), and atropine administration improved cardiac output, stroke volume, heart rate and organ perfusion. ChE activity was low after Cl_2, unchanged with atropine.

Conclusion: High level Cl_2 exposure causes multi-organ failure and high mortality. Atropine improved survival, improved cardiac output and perfusion, and improved blood acidosis. However, it did not improve hypoxemia. Atropine should be considered as acute therapy for high level Cl_2 inhalation.

Establishment of a method for generating human induced pluripotent stem cells (iPS)

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In 2012 the Nobel Prize was given to John B. Gurdon and Shinya Yamanaka for their breakthroughs in generating iPS (induced pluripotent stem cells) from adult cells. The opportuni-

ties are quite obvious, as adult cell material can be dedifferentiate and redifferentiate into any other cell type with the exact same genotype. Examples of future application could be, e.g., organ transplantation, drug testing, basic research to name a few. The reprogramming is getting possible by transduction of four exogenous reprogramming factors. This allows the production of cells in an embryonic like state without the ethical concerns. In our approach the four factors, Oct3/4, Sox2, c-Myc, and Klf4, were purchased from addgene transfected in E.coli. After purification plasmids were used to transfect adult fibroblasts via electroporation using the Lonza Nucleofector System. Successful reprogramming was proven by GFP as a marker, morphology of the cells and RT-PCR.

By modifying different variables (exposure to the transfection solution, time of the passage, passage solution) we were able to establish a protocol for reprogramming human dermal fibroblasts into iPS with a success rate of 10 %. The clones received from this procedure can be kept in media for around 6 weeks.

Furthermore, forced spontaneous redifferentiation of these cells resulted in neuronal cells. This was proven with antibodies by immunhistochemistry (e.g. POU2F1 shows early neuroectodermal cells, PAX-6 visualizes a protein ensuring the process of neurogenesis or, at a later stage, synapsin marking the nerve terminal of axons, specifically the membranes of synaptic vesicles).

The method of generating iPS has been established successfully. After differentiation of the iPS, early neuronal cells as well as mature neuronal cells could be identified. Even synaptic vesicles could be observed.

New concept for the neutralization of irritant and corrosive chemicals on the skin and on clothing.

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Objective: Contact of irritant or corrosive chemicals with the skin is possible during military operations or terrorist attacks. CBRN units, hospitals and disinfection units also have large stocks of aggressive chemicals. In case of accidents these must be neutralized. Showers or large amounts of water are often not available.

Approach: We have developed a general concept that allows physicians and first responders to react quickly and effectively to such situations. The kit developed here consists of a double-chamber-spray with two different solutions: The first solution consists of an amino acid buffer and a calcium salt in water, the second solution consists of a chemical reducing agent. When the spray head is actuated, the two liquids mix and can be sprayed onto the affected areas.

Results: The product was tested *in vitro* with the following high concentrated chemicals: nitric acid, sulfuric acid, hydrochloric acid, peracetic acid, sodium hypochlorite, calcium hypochlorite, sodium hydroxide, ammonia, hydrofluoric acid and formaldehyde. These chemicals were mixed with the freshly mixed product in a ratio of 1 : 30 and temperature, redox potential and pH value were monitored over 30 minutes. Strong acids and bases were neutralized immediately. Formaldehyde was bound as imine. The redox potential of oxidants decreased significantly. Fluoride precipitated within several minutes as calcium fluoride

Conclusion: We have developed a kit, which can be used independently of water or power supply. The double-chamber spray is stable at room temperature for several years and ready to use in any situation. The concept is highly effective for all irritant and corrosive chemicals on skin and clothing as well as on the surface of small items.

Acute intoxications with a novel synthetic opioid, U-47700

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Background: Since 2013, the European Monitoring Center for Drugs and Drug Addiction (EMCDDA) reported a growing number of new psychoactive substances (NPS), reaching 100 new substances annually in the last years. Among them synthetic opioids represent a relatively small group. U-47700 (3,4-dichloro-N-[2-(dimethylamino)cyclohexyl]-N-methylbenzamide) is a very potent and selective μ-opioid agonist reported as an NPS for the first time in October 2014 in Europe.

Methods: Cases were from the Poison Centre (PC) Freiburg and from a prospective observational study of patients treated in emergency departments (ED) after the intake of SC and other NPS, in cooperation with the Institute of Forensic Medicine Freiburg. Clinical features and follow-up information were gathered by a structured questionnaire for physicians. Serum and/or urine samples of ED patients were analyzed using liquid chromatography-electron spray ionization-tandem mass spectrometry (LC-ESI-MS/MS) screening methods for NPS.

Results: Four male patients (2027 years) were included. The first case was reported in June 2016: A 24-year-old male was found unconscious with bradypnoea. Emergency medical services were contacted by laymen, who started cardiopulmonary resuscitation (CPR). However, the patient did not survive. Three other male patients with deep coma, miosis and respiratory insufficiency (bradypnoea or apnoea, respiratory acidosis) were given intravenous naloxone by emergency physicians, after which the patients' respiration improved. All three patients were stabilized further in the emergency department. However, one patient developed acute renal failure. Serum and/or urine samples were available in two of the three ED patients, and the intake of U-47700 was analytically confirmed.

Conclusion: Since 2016, acute intoxications with U-47700 have been reported in Germany. U-47700 is a very potent μ-opioid agonist. Because of its high potency and rapid onset of action, the use of U-47700 implies the risk of potentially lifethreatening overdose. Fatal poisonings have also been reported from other European countries. The availability of highly potent opioids via Internet shops is alarming.