Title

Development of an acute, short-term exposure model for phosgene

Journal

Toxicology Mechanisms and Methods, 29(8)

ISSN

1051-7235

Authors

Hobson, Stephen T
Casillas, Robert P
Richieri, Richard A
et al.

Publication Date

2019-10-13

DOI

10.1080/15376516.2019.1636170

Peer reviewed

Published in final edited form as:

Toxicol Mech Methods. 2019 October ; 29(8): 604–615. doi:10.1080/15376516.2019.1636170.

Development of an acute, short term exposure model for phosgene.

Stephen T. Hobson[1,2], **Robert Casillas**[3], **Richard A. Richieri**[1], **Robert N. Nishimura**[5,6], **Richard H. Weisbart**[5,6], **Rick Tuttle**[4], **Glenn T. Reynolds**[1], **Missag H. Parseghian**[1,*]

[1]Rubicon Biotechnology, 5753-G Santa Ana Canyon Road, #532, Anaheim, CA, 92807

[2]Liberty University, Dept. of Biology and Chemistry, 1971 University Blvd., Lynchburg, VA, 24515

[3]Latham BioPharm Group, 101 Main St., Cambridge, MA, 02142

[4]MRIGlobal, 425 Volker Boulevard, Kansas City, MO 64110-2241

[5]University of California, Los Angeles, School of Medicine, Los Angeles, CA 90095

[6]Veterans Affairs Greater Los Angeles Healthcare System, Los Angeles, CA 91343

Abstract

Phosgene is classified as a chemical warfare agent, yet data on its short-duration high concentration toxicity in a nose-only exposure rat model is sparse and inconsistent. Hence, an exposure system for short term/high concentration exposure was developed and characterized. Herein, we report the median lethal concentration (LC_{50}) for a 10-min nasal exposure of phosgene in a 24-h rat survival model. Male Wistar rats (Envigo) weighing 180-210 g on the day of exposure, were exposed to phosgene gas via nose-only inhalation using a system specifically designed to allow the simultaneous exposure and quantification of phosgene. After 24 h, the surviving rats were euthanized, the lung/body mass ratio determined, and lung tissues analyzed for histopathology. Increased terminal airway edema in the lungs located primarily at the alveoli (resulting in an increased lung/body mass ratio) coincided with the observed mortality. An LC_{50} value of 129.2 mg/m^3 for a 10-min exposure was determined. Furthermore, in agreement with other highly toxic compounds, this study reveals a LC_{50} concentration value supportive of a non-linear toxic load model, where the toxic load exponent is > 1 (n_e = 1.17). Thus, in line with other chemical warfare agents, phosgene toxicity is predicted to be more severe with short-duration, high-concentration exposures than long-duration, low-concentration exposures. This model is anticipated to be refined and developed to screen novel therapeutics against relevant short-term high concentration phosgene exposures expected from a terrorist attack, battlefield deployment, or industrial accident.

Short Abstract

[*]**To whom all correspondence should be addressed Missag H. Parseghian, PhD**, Chief Scientific Officer, Rubicon Biotechnology, 5753-G Santa Ana Canyon Road, #532, Anaheim, CA, 92807 USA, Phone: 1-818-422-5734, mparseghian@rubiconbio.com.

Disclosure of interest

MHP, RAR, and STH are compensated by Rubicon Biotechnology. MHP, RAR and GTR are co-owners of Rubicon.

Data on phosgene's short-term high concentration toxicity in a rat model is sparse. Using a system designed for short term high concentration exposures, the median lethal concentration (LC_{50}) for a 10-min nasal exposure of phosgene in a 24-h rat survival model of 129 mg/m^3 (31.5 ppm) was determined. Our study reveals a LC_{50} concentration value supportive of a non-linear toxic load exponent model. Hence, like other chemical warfare agents, phosgene toxicity is predicted to be more severe with short-duration, high-concentration exposures than long-duration, low-concentration exposures.

Keywords

Phosgene; Acute Toxicity; Lethal Dose; Lethal Concentration; Defense Countermeasures; Toxic Load Exponent; Toxic Load Model

Introduction

Phosgene (carbonyl chloride), a toxic colorless gas at room temperature, is used widely in industry in the synthesis of plastics, dyes, pharmaceuticals, and agro-chemicals. Its ready availability and high toxicity make it an agent of concern to the military and to homeland security authorities (Bast and Glass-Mattie 2015; Baggett and Simpkins 2018). Indeed, during World War I the greatest number of fatalities was caused by phosgene (Summerhill et al. 2017). Developed as a war gas by Fritz Haber, his post-war studies on acute lethality led him to suggest a relationship, now known as Haber's rule, where the physiological effects of phosgene and other toxic gasses are proportional to the concentration and duration of exposure ($C \times t$) (Haber 1924). As an aside, this was not a new toxicological observation, having been first proposed by Warren (1900) in studies on the toxicity of salt solutions that kill the microscopic planktonic crustacean, *Daphnia magna*.

Despite phosgene's industrial prevalence, there is no FDA-licensed therapeutic treatment for toxic phosgene exposure (Sciuto and Hurt 2004; Russell et al. 2006; Smith et al. 2009; Summerhill et al. 2017). Two characteristics give phosgene greater lethality: density and odor. It is denser than air, thus it can accumulate in low-lying areas and not dissipate quickly (Bast and Glass-Mattie 2015). Its odor, or lack thereof, is mild with a threshold for human detection between 0.5 and 1.5 ppm, yet damage to the lower respiratory tract can already occur at lower concentrations (Bast and Glass-Mattie 2015). Initial exposure may result in ocular, nasal, and throat irritation; however, the insidious nature of the pathogenesis is characterized by a 'latent' phase (Diller 1985), a period of 2-24 h in which victims appear symptom free (Russell et al. 2006; Smith et al. 2009). The duration of the latent phase appears inversely proportional to the exposure dose (Public Health England 2016). The National Institute for Occupational Safety and Health's (NIOSH) *Immediately Dangerous to Life or Health* (IDLH) level for phosgene has been set at 2 ppm (8 mg/m^3) (https://www.cdc.gov/niosh/idlh/75445.html). Inhalation of a high dose (>600 mg/m^{3}x min), results in neutrophil infiltration, pulmonary edema and oxidative stress in the lungs (Ghio et al. 1991; Russell et al. 2006). Based on observations from workplace accidents, inhalation of higher doses (≥1200 mg/m^3 × min) may also result in mortality (Diller 1985).

Previous attempts to curb neutrophil infiltration using cyclophosphamide, the 5-lipoxygenase inhibitor AA861, or the microtubular poison colchicine have been unsuccessful (Ghio et al. 1991); furthermore, the use of a nebulized beta-agonist, salbutamol, has shown a deleterious effect on arterial oxygenation (Grainge et al. 2009). Neither treatment with nebulized or intravenous steroids (Smith et al. 2009; Grainge and Rice 2010) nor the use of the diuretic furosemide to reduce lung edema improves survival (Grainge, Smith, et al. 2010). Other treatment strategies such as angiopoietin-1(He et al. 2014), NOS-2 inhibitors (Filipczak et al. 2015), and ulinastatin (Shen et al. 2014) have only shown efficacy in small animal studies. The efficacy of a prophylactic dose of ibuprofen, given 30 min prior, for the reduction of observed lung edema in phosgene exposed rats has also been reported (Sciuto et al. 1996), although such a pre-treatment strategy is unrealistic given the nature of “no-warning” chemical exposure events, like those expected on the battlefield, a terrorist attack, or workplace accident. Providing victims with supplemental oxygen appears to be the only treatment that results in improved survival, improved arterial oxygenation and reduced lung edema (Russell et al. 2006; Grainge, Jugg, et al. 2010). Following exposure to hazardous chemical fumes, survival is improved by the preservation of cellular function in the lungs.

Our laboratory is developing therapeutics that involve intracellular delivery of a targeted heat shock protein (Hsp72) to damaged lung tissue to reduce the destructive effects of oxidative stress and inhibit induction of apoptosis (Parseghian et al. 2016). Hence, it is imperative we have animal models designed for acute exposure and for the post-exposure treatment of both acute short-term phosgene inhalation and the subsequent 24-h latent injury phase. To that end, a 10-min phosgene nose-only exposure in rats with a 24-h survival study was initiated because no definitive screening model exists. Furthermore, although extensive studies of chronic low-concentration phosgene exposure suggested a linear C x t relationship in rats exposed for 30, 60 and 240 min using a nose-only inhalation system (Pauluhn 2006a), the toxicity values from 10-min exposures under the same circumstances is not consistent among investigators (Zwart et al. 1990; Pauluhn 2006a). The model reported here addresses the high concentration exposure at ground zero caused by phosgene’s greater atmospheric density and the realistic response of victims who are not immediately inclined to escape due to the mild odor characteristics. Our model provides toxicity values for the 10 min exposure that better align with the trends seen for longer exposures in an earlier study (Pauluhn 2006a) while revealing that the risk of acute high concentration phosgene exposure has been under-predicted by others (Zwart et al. 1990; Pauluhn 2006a). The results from this study demonstrate that phosgene’s toxicity, like some other chemical warfare agents, does not strictly adhere to the linear relationship ascribed to Haber’s rule when faced with short duration, high concentration exposures.

Materials and Methods

Test Materials

Tanked phosgene gas at a concentration of one percent with a certified purity of 1.000 ± 0.02% were obtained from Custom Gas Solutions (Durham, NC). Ketamine-HCl and xylazine-HCl were purchased from Sigma (St. Louis, MO). Euthasol® [a cocktail of 390

mg/mL sodium pentobarbital and 50 mg/mL sodium phenytoin] was obtained from Virbac Corporation (Fort Worth, TX). Phosgene is a highly toxic agent and must be handled with extreme caution. Phosgene was handled and used in accordance with safety and surety procedures in a US Army approved laboratory at MRIGlobal (Kansas City, MO).

Animal Model

All animal studies were reviewed and approved by MRIGlobal's IACUC, and conducted at their Kansas City, MO facility on behalf of Rubicon Biotechnology. All studies were conducted according to AAALAC guidelines and all animals received humane care in compliance with MRIGlobal's guidelines, and as outlined in the Guide for the Care and Use of Laboratory Animals published by the National Institutes of Health.

Male Wistar rats (strain Hsd:WI, Harlan Laboratories/Envigo), ≥ 6 weeks old and 180-210 g were used on the day of phosgene exposure. Only male rats were used since we did not anticipate sex differences for this acute exposure scenario and were randomly assigned to exposure groups. A 24-h survival endpoint was identified as this model was designed to screen novel therapeutics against relevant short-term high concentration phosgene exposures consistent with real-world human accidental exposures that progresses through a 24-h latent injury phase followed by high morbidity and mortality (Diller 1985; Public Health England 2016). All animals were weighed within 24 h prior to phosgene exposure. General health observations were recorded daily throughout the quarantine and acclimation period, just prior to phosgene exposure, a minimum of twice on the day of phosgene exposure, and at least once on the day of euthanasia. Cage side observations included hunched posture (kyphosis), dehydration, rough coat (ungroomed hair), and inappetence (anorexia); these observations are industry standards for toxicology studies (Fentener van Vlissingen et al. 2015). Time of death was documented, and following death, a body weight was recorded, the lung was harvested, and weighed for determination of lung/body weight ratios.

Physiological and Histological Analyses

The left side of the lung was fixed for histological analysis by first ligating the right primary bronchus, inflating the left side with 10% formalin via the trachea, and then ligating the trachea. The right lung was removed for further processing (see below), while the left lung was placed into a macrocassette labeled with the animal ID, and then placed into a large 10% formalin container for fixation (10:1 volume ratio of formalin to tissue). After the tissue had been fixed for a minimum of 72 h, cassettes were transferred to heat sealed bags for shipment to the board-certified animal pathologist. Upon receipt, tissues were alcohol dehydrated, xylene cleared, paraffin embedded, sectioned and then stained with hematoxylin and eosin for conventional light microscopy. Sections were analyzed and photographed on an Olympus IMT-2 microscope and digital images captured using the MagnaFIRE SP software (version 1.0 ×5; Optronics Corporation). All images in Figure 5 were photographed through an Olympus SPlan10PL 10X objective lens, a 1.5X stage lens and a 10X camera lens, resulting in a 150X magnification.

For the dose range studies with phosgene, the entire right side of the lung (all three lobes) was snap frozen in liquid nitrogen and stored at −80°C until analysis of oxidative stress. For

the LC_{50} confirmation survival study discussed in Figure 6, the right side of the lung was bisected, and the superior lobe weighed. This section of tissue was then placed on to aluminum foil in a 100°C oven and baked for 8 days before being re-weighed in order to determine the extent of pulmonary edema in the original tissue sample. (Parker and Townsley 2004). The middle and inferior lobes of the right lung were frozen in liquid nitrogen and stored at −80°C for future analysis.

Phosgene Exposure

The underlying principle guiding our short-term high concentration animal model is simulation of exposure at ground zero following a mass casualty event from an intentional release or catastrophic accident involving phosgene. Thus, while our exposure system design is similar to previous work (Pauluhn 2006a), the geometry of the exposure system does differ. We designed a gas delivery system and used an exposure system for our purposes of developing a relevant animal model for short-term high concentration phosgene. A CH Technologies (Westwood, New Jersey) 5 port nose-only inhalation system was used to expose the rats to phosgene gas (Figure 1). The exposure system is unique in that the system displacement volume is extremely small, with an internal volume of approximately 200 cc. This design facilitates exposure initiation concentration, increase to stability, and concentration decay or purge time of the system following exposures to be rapid at low system operation flows. Exposures were conducted at a total flow rate of 5 L/min, with 1 L/min being delivered to each rat during the exposure process. This provided approximately 25 total volume displacements and regeneration of fresh phosgene gas per minute to the system and breathing zones of the animals. This high throughput and replenishment of challenge gas in the breathing zone of the animals assures that respiration byproducts (CO_2), and the potential of rebreathing resident gas, is eliminated. This small displacement volume also assured gas concentration increase and stability to be achieved within 10-15 s following the initiation of phosgene gas generation and delivery to the system as well as purging of resident gas following exposure. This feature provides a more linear concentration delivery and accuracy response over time at the same operational flow rates when compared to larger displacement exposure systems that require a longer time for concentration increase and stability following the initiation of the gas challenge, and an increased time to purge of resident gas following the termination of exposures.

The exposure system has capacity to expose up to five animals simultaneously (Figure 1A). The top and bottom of the exposure system are ported for the introduction of the inhalation challenge gas and exhaust of resident gas and respiration byproducts with the system. The system was operated in a push/pull dynamic flow mode. Phosgene gas flow rates introduced to the system were provided via a pressure regulated 1% $COCl_2$ gas, ± 0.02% certified purity cylinder (Customgas Solutions, Durham, NC) and were flow controlled with a corrosive gas resistant valved flow meter (Dwyer Instruments, Michigan city, IN). Phosgene gas flow was introduced through chemically inert Teflon PTFE tubing with additional high purity tanked and regulated zero grade dilution air (Praxair Inc., Kansas City, MO). The flow meters were verified for flow rate control and accuracy using a calibrated NIST traceable standard Mini - BUCK Calibrator and flow rates in relation to meter scale readings were plotted for flow rate setting reference for conduct of the study. The dilution air flow rates

were metered using a mass flow meter (Omega Engineering, Inc. Stamford, CT) and converged and mixed at a junction upstream of an FTIR concentration measurement instrument, and the exposure system. Phosgene gas and zero air dilution flow rates were controlled at ratios applicable to achieving the desired inhalation exposure concentration (Table 1). Challenge gas was delivered in a flow through (push/pull) mode with system inlet and exhaust flow rates balanced for a constant replenishment of fresh challenge gas in the respiration zones of the rats. System challenge and exhaust flow rates were operated at equilibrium with the system maintained at ambient pressure conditions in relation to the hood line, and system operating pressures were monitored using a ± 0-0.5 inches H_2O Magnehelic differential pressure gauge (Dwyer Instruments, Michigan City, IN) for all conducted exposures. Resident phosgene gas and animal respiration byproducts were exhausted through a series of sodium hydroxide scrubbers before release into the hood line.

Five tubes, each containing a rat, were attached to the exposure ports of the system for each round of experimentation. After delivery of phosgene gas and removal of the rats from the system, the exposure tubes were decontaminated with 10% sodium hydroxide (NaOH), rinsed with water, and dried for reuse between exposures. All exposures were conducted at room temperature.

The starting concentration in the compressed cylinder for phosgene was 40,477 mg/m^3 with the balance nitrogen. Since 4.1 mg/m^3 equals 1 ppm (Li and Pauluhn 2014), the starting concentration equaled 10,000 ppm (hence, 1% phosgene). Both the phosgene and compressed air tank were regulated to provide a head pressure of 35 psi to independent valve-controlled flow meters. Prior to delivery to the rats, the phosgene challenge gas concentration was targeted by adjusting the compressed zero air dilution flow rate and analyzing the phosgene gas concentration using a Thermo Scientific, Nicolet model IS10 Fourier Transform Infra-Red (FTIR) spectrometer, with a 2-liter displacement 10m path length gas cell, to verify accuracy of the exposure concentration (Figure 1B). Concentrations were also measured following each exposure trial to verify the delivered stability of the gas concentration during exposures (Table 1).

Phosgene gas flow rates and additional zero air dilution flow rate ratios were derived based on the total system flow rate delivered to the exposure system, and the certified purity concentration of the phosgene gas tank. The FTIR was located in-line between the gas generation and exposure system components for exposure concentration verification. Initial calibration of the FTIR was conducted by purging the gas cell with zero air and analyzing the zero air for purity (no impurities detected), and baseline calibration settings of the unit. Following zero air analysis, volumetric injections of neat 1% phosgene were drawn from a reference septum of the cylinder gas delivery tubing using a graduated gas tight syringe. Syringe samples were injected through a septum into the FTIR gas cell for quantitative analysis of the phosgene concentration related to the instruments IR peak response. Additional calibration of the FTIR included flow through analysis of the phosgene challenge gas over a range of flow meter regulated zero air gas dilution flow rates. Five-point FTIR calibrations were conducted and linear regression plotted preceding each exposure trial for defining to verify phosgene gas and dilution flow rate settings, and the phosgene challenge concentration vs the FTIR peak height response. Prior to the initiation of each exposure and

phosgene gas concentration measurement, the FTIR gas cell was purged with zero air to eliminate any residual phosgene gas or potential background interference.

Rats were acclimated by placement in the exposure tubes for 10 min two days prior to exposure (Study Days −2 and −1) to minimize stress due to confinement in exposure tubes. For nose-only studies, acclimation is a common practice (Sweeney et al. 2015) and studies have been conducted investigating stress levels in rats and mice confined to an exposure tube for up to 4 h (Narciso et al. 2003) (Figure 1C). In MRIGlobal's experience with rats and similar nose-only exposure studies conducted with other toxic inhalants, the 2-day acclimation reliably provides minimally stressed (calm) rats (based on clinical observation) at start of the inhalation exposure. On Study Day 0, rats were loaded into exposure tubes just prior to the phosgene challenge. Post-exposure, rats were transferred to a holding cage within the hood for 10 min to allow surface off-gassing of residual phosgene, then transferred to cages in the laboratory (outside of the hood line). At the end of 24 h post-exposure rats were euthanized by a standard, IACUC approved protocol.

Statistical Analysis.

The sigmoidal dose-response curve resulting from the analysis of phosgene concentration versus survival was transformed into a linear regression analysis using probit analysis (Finney 1952). Comparison of the lung / body weight ratios presented in Table 2 and graphed in Figures 4 and 6B used the unpaired Student's t-test analysis ($P \leq 0.05$) available through the Prism GraphPad 5 software package.

Results

Inhalation of increasing concentrations of phosgene at a 10-min exposure time resulted in increased mortality (Figure 2). Probit analysis of the data subset where a dose response curve could be generated (94.6 mg/m^3 to 189.6 mg/m^3; 23 ppm to 46 ppm), resulted in a calculated LC_{50} of 129.2 mg/m^3 (31.5 ppm) for a 10 min exposure (Figure 3). The 95% confidence limits were 109.2 and 145.7 mg/m^3 (26.6 and 35.5 ppm). At 24 h after phosgene challenge, surviving rats were euthanized, and all rats, including those that died during the 24 h period, were weighed. Lungs from all rats were harvested and weighed to determine the lung/body mass ratio, an indirect measure of pulmonary edema and extravasated protein in the bronchoalveolar lavage fluid (BALF) (Pauluhn 2006b). Lungs from rats exposed to greater concentrations of phosgene had statistically significant ($P \leq 0.05$) higher lung/body mass ratios when compared to the cohort exposed to the lowest phosgene concentration (94.6 mg/m^3; Table 2). Rats that survived 24 h after phosgene exposure showed some red mottling of the lung tissue characteristic of pulmonary hemorrhaging; however, rats that died much earlier had enough hemorrhaging to visibly change lung coloration from tan to burgundy or dark red upon gross observation (Figure 4).

Histological analysis of 10% formalin fixed lung tissue, sectioned and stained with hematoxylin and eosin (H&E), confirmed hemorrhaging was greater in those rats that succumbed prior to the 24 h time point (Figure 5A). Samples were blinded prior to scoring by a board-certified veterinary pathologist (see Figure 5 legend for definition of scores). For all rats exposed at 134.6 mg/m^3 (the cohort closest to the calculated LD_{50} of 129.2 mg/m^3;

n=5), the primary lesions observed were airway exfoliation, terminal airway edema, and the presence of fibrin strands (Figure 5B). At this exposure level, two of the three rats that died prior to the 24 h time point had complete exfoliation of the airway epithelium while the others showed minimal vacuolar degeneration. These same two rats also had diffuse pulmonary congestion reflecting an engorgement of blood vessels and capillaries in the interstitium. Terminal airway edema was moderate but dispersed in all rats exposed at this concentration and fibrin strands were seen in most of the lesions along with the occasional presence of macrophages. Other lesions observed at the 134.6 mg/m^3 (32.8 ppm) cohort were perivascular or interstitial edema, reflecting expansion of the connective tissue surrounding larger pulmonary blood vessels, and multifocal alveolar or parenchymal edema in a couple samples, reflecting accumulation of eosinophils.

To confirm the calculated LC_{50} of 129.2 mg/m^3, an additional nine rats were challenged at that phosgene concentration for 10 min and survival was determined over the next 24 h (Figure 6A). Based on FTIR measurements, 100.5-100.6% of the targeted phosgene was delivered (i.e. 129.8 – 130 mg/m^3), with 3 out of 9 rats (33.3%) surviving. Lung / body weight ratios were comparable to the ratios obtained earlier for 134.6 mg/m^3 phosgene (Figure 6B).

Discussion

As a prerequisite to screen therapeutics for acute inhalation of phosgene, we needed to establish a consistent short-term rat nose-only inhalation toxicity 24-h survival model that better reflected high concentration exposures expected to occur at ground zero. Since highly toxic chemicals like phosgene demonstrate dramatically steep slopes in toxicity dose-response curves with small increases in dose, the variability in previously reported 10-min exposure toxicity values (Zwart et al. 1990; Pauluhn 2006a) is not unexpected and is very likely due to even slight differences in the design/plumbing of inhalation systems, animal strain, and survival endpoint selection (e.g., 4-h, 24-h, or 14-d). With our system designed for acute (10 min) phosgene exposure, the calculated LC_{50} is 129.2 mg/m^3 (31.5 ppm; LCt_{50} = 1292 mg/m^3 × min). An earlier study by Pauluhn (2006a) investigated four concentrations at the 10 min exposure time: 166.5, 181.6, 212.0 and 250.9 mg/m^3 whereas the current study reported herein addressed a broader concentration range (Figure 2, Table 1). The Pauluhn study reported that a 10 min exposure at 181.6 mg/m^3 resulted in 100% survival, yet in this study none of the rats survived a 10 min exposure at 172.5 mg/m^3. Furthermore, our study showed a normal dose-response curve compared to that earlier study (Figure 2). Pauluhn calculated the LC_{50} for a 10 min exposure at 253.3 mg/m^3 (62 ppm; LCt_{50} = 2533 mg/m^3 × min).

A much earlier investigation of 10 min phosgene exposures by Zwart et al. (1990) studied concentrations ranging from 50 – 424 mg/m^3. Both rats and mice were studied by exposing 5 males and 5 females from each species at each phosgene concentration. In that study, the authors report a higher calculated LC_{50} for a 10 min exposure in rats at 334 mg/m^3 (81ppm) and further report separate LC_{50} values for male mice (322 mg/m^3; 79 ppm) and female mice (244 mg/m^3; 60 ppm).

We note different strains of rats were used in each study, which likely play a role in the variation in toxicity seen. The Zwart et al. (1990) study used Wistar derived rats ranging in mass from 150 – 170 g for males and 130 – 140 g for females. The Pauluhn (2006a) study used the Cpb:WU strain of Wistar rats derived from the Harlan Sprague Dawley (Hsd) line, whereas in our study we used a different strain of Wistar rats (Hsd:WI) based on animal availability. Pauluhn's phosgene study also used male and female rats ranging in mass from 184 – 209 g and 160 – 180 g, respectively. Our study used only male rats with a mean body mass of 180-210 g, therefore, the body mass of male rats in this study and Pauluhn's were comparable.

As for differences in the exposure systems, Zwart et al. (1990) placed rats in custom-made horizontal glass cylinders and attached them to sample ports. The directed-flow nose-only system assembled by MRIGlobal for our study while similar to the one described in Pauluhn (2006a) has several distinct features specific to this system that were designed to meet future regulatory compliance criteria for evaluating phosgene medical countermeasures in accordance with the FDA's Animal Rule (Krishna and Goel 2015). (Figure 1).

Two key factors differentiate our study from the earlier ones, the first being the acclimation of rats to the confining environment of the exposure tubes for 10 min on Study Days −2 and −1 to minimize stress levels and better mimic the early stage behavior of exposure victims at ground zero. Although we did not assay for stress biomarkers, our clinical observations of the rats' behavior prior to and during the 10-min exposure did not indicate undue stress. It is important to note, that in Pauluhn's studies, the lower toxicity (10 min LC_{50} of 253.3 mg/m^3) and the unusual survival curve (Figure 2) was attributed to "an initial and concentration-dependent increase in the apnea time, accompanied by decreased respiratory minute volumes" which resulted in greater survival rates than expected for the rats, and in turn complicating the analysis (Pauluhn 2006a). This phenomenon is described as a reflexive behavior in rodents and canines caused by phosgene's stimulation of vagal C-fibers innervating the lower airways and controlling spontaneous breathing (Li and Pauluhn 2014). As a consequence of Pauluhn's observations, future work in his group was conducted with 30-min or greater exposure times to ensure consistent results (Pauluhn 2006b). Some of the lower toxicity seen in the Pauluhn (2006a) study may have also been caused by induction of stress proteins during confinement of unacclimated rats that resulted in mitigation of apoptosis and inflammation.

A second factor that differentiates our study from Zwart or Pauluhn, is in the design of the exposure system. First, the use of a FTIR spectrometer accurately quantitates the delivered phosgene concentrations. None of their studies used such instrumentation; however, Pauluhn (2006a) does use a soap-bubble meter to monitor flow meter accuracy. Second, our system was designed to rapidly deliver the concentration of the calculated phosgene in a step function rather than gradually increase the concentration (*vide supra*). Our initial study was designed to accurately deliver phosgene concentrations ranging from 191 to 316 mg/m^3 over 10 min (Table 1, Study 1) to replicate these earlier studies; however, at these concentrations in this system, no rats survived to 24 h. Subsequent studies with reduced phosgene concentrations (94.6 to 189.6 mg/m^3) were then performed (Table 1, Study 2). The number of rats in each cohort are listed in Figure 2 and Table 1. A Probit Analysis is the preferred

method of analyzing a dose response curve when there is a binomial response (Finney 1952); in this study, the binomial response being survival at 24 h (survived vs died, Figure 3). The method allows for transformation of a sigmoidal curve to a linear one for regression analysis. Probit Analysis was conducted on the data in our dose response curve where survivability was >0% resulting in a LC_{50} of 129.2 mg/m^3 (95% confidence limits of 109.2 and 145.7 mg/m^3) compared to earlier work where LC_{50} was 253.3 mg/m^3 and 95% confidence limits were 194 – 331 mg/m^3 for Pauluhn (2006a) and LC_{50} was 334 mg/m^3 and 90% confidence limits were 306 – 363 mg/m^3 for Zwart et al. (1990).

Phosgene's poor water solubility and rapid hydrolysis causes its reaction with the mucus layer in the upper respiratory tract to be largely irritating (Borak and Diller 2001). In contrast, toxicity is confined to the lower respiratory tract, including the alveolar-blood barrier (Pauluhn 2006a), resulting in inflammation, pulmonary edema and oxidative stress (Chen et al. 2011; Li and Pauluhn 2014). The resultant increased fluid and protein flux into the lavage generally indicates serious lung injury (Parker and Townsley 2004). Our results further confirm these observations where all survivors at 24 h had lung / body weight ratios ≤ 0.02 (Figure 4). An unpaired Student's t-test analysis of ratios obtained at each exposure and compared to the lowest phosgene concentration (94.6 mg/m^3) finds the increased ratios to be statistically significant ($P \leq 0.05$) for all exposures above 134.6 mg/m^3 (Table 2). Hence, there is a significant correlation between lung / body weight ratio (Figure 4) and survival (Figure 2) at 10 min phosgene exposures. Furthermore, our calculated LC_{50} determination of 129.2 mg/m^3 (31.5 ppm) closely matches the pulmonary edema reported during the 24 h latent period for exposures of more than 30 ppm-min (Bast and Glass-Mattie 2015).

Earlier reports determined a linear adherence of phosgene supportive of Haber's rule (C x t = toxic load dosage). Such a linear correlation allows for normalization of the concentration data when multiplied by time of exposure and is often presented as LCt_{50}. Such a correlation also means that lower phosgene concentrations delivered for longer periods of time are just as toxic as higher phosgene concentrations delivered in shorter time. Zwart et al. (1990) determined similar LC_{50} values for a 30 min exposure at 84 mg/m^3 (LCt_{50} = 2520 mg/m^3 × min) and for a 50 min exposure at 49 mg/m^3 (LCt_{50} = 2450 mg/m^3 × min). However, these values correlate poorly with the 10 min exposure LC_{50} value of 334 mg/m^3 (LCt_{50} = 3340 mg/m^3 × min). Similarly, the Pauluhn (2006a) results determine the LC_{50} for a 30 min exposure to be 54.5 mg/m^3 (LCt_{50} = 1635 mg/m^3 × min), a 60 min exposure to be 31.3 mg/m^3 (LCt_{50} = 1878 mg/m^3 × min), and for a 240 min exposure to be 8.6 mg/m^3 (LCt_{50} = 2064 mg/m^3 × min). These values are in contrast to the 10 min LC_{50} of 253 mg/m^3 (LCt_{50} = 2530mg/m^3 × min). In both earlier studies, acute phosgene exposure for 10 min was reported to be less toxic than our data show. The earlier studies were both described as 14-day survival studies, with one study reporting all 10 rats surviving exposure of 181.6 mg/m^3 for up to 2 weeks (Pauluhn 2006a). In contrast, our system reaffirms the steep toxicity curve for phosgene, which has been characterized by others as being so steep that it approaches a step function (Pauluhn 2006a). Based on statistical analysis, we ascertained the LC_{50} for a 10 min exposure of phosgene to be 129.2 mg/m^3 (LCt_{50} = 1292 mg/m^3 × min); this result better aligns with the trends reported by Pauluhn (2006a) at 30, 60 and 240 min (Figure 7A).

A second experiment confirmed the 10-min data and resulted in similar survival curves (Figure 6A) and lung / body weight ratios (Figure 6B) to the 134.6 mg/m^3 dosage. Indeed, there was no statistical difference for survival at 24 h post-exposure ($P = 0.8209$) or for the lung / body weight ratios ($P = 0.8044$) between the 134.6 mg/m^3 and the 129.2 mg/m^3 cohorts.

When we compared our LC_{50} for a 10 min exposure with those obtained by Pauluhn for 30, 60- and 240-min exposures (Figure 7A), it quickly was apparent the LCt_{50} dosages trended higher with increased exposure times (Figure 7B) rather than being about equal, as one would expect under Haber's rule. This non-linear trend has been seen with other chemical warfare agents such as sarin [Spruit et al. 2000; Mioduszewski et al. 2002] and cyanide [Sweeney et al. 2015] in which a short-duration, high-concentration exposure appears to be more toxic than a long-duration, low-dosage exposure. We hypothesized this may be the case for phosgene as well. Our results for the 10 min exposure, when combined with the longer exposure data from Pauluhn (2006a), confirms this hypothesis. We believe our toxicity values for the 10 min exposure are more accurate, given the tighter 95% confidence limits.

Ironically, there is no indication that Fritz Haber believed C x t was a linear phenomenon for all toxicants or that it held linearly for long periods of exposure given an organism's toxicokinetics and detoxification mechanisms (Miller et al. 2000; Rozman and Doull 2000). Researchers now view C x t as a special case in a family of power law curves that help determine the toxic response from concentration and time of exposure values (Miller et al. 2000). The phenomenon of non-linearity in toxicity is mathematically described by replacing the linear C x t = dosage determination of Haber's rule with a "toxic load exponent" n_e, where $C^{ne} \times t = TL$, where TL is the toxic load dosage (ten Berge et al. 1986). The exponent is determined through animal experiments at several exposure concentrations or durations. In this case, we used LC_{50} as the parameter to determine the exponent, hence $C^{ne} \times t = TL$ becomes $(LC_{50})^{ne} \times t = TL_{50}$ (Figure 7C). If the value of the exponent is >1, the short-duration, high-concentration exposures are more toxic; however, if the exponent is <1 for an agent, then a short-duration, high-concentration exposure is not as toxic as a long-duration, low concentration one. Plotting exposure time versus the LC_{50} on a logarithmic scale allows for a regression analysis to determine the value of n_e (Weinrich et al. 2008). Here, we used the method of converting both sides of the equation to log10 before plotting (Sweeney et al. 2015), hence:

$$\text{Log}[(LC_{50})^{ne} x\, t] = \text{Log}\, TL_{50} \text{ becomes}$$
$$\text{Log}\,(t) = -n_e \,\text{Log}(LC_{50}) + \text{Log}\, TL_{50}$$

The negative slope of the linear equation generated in the graph (Figure 7D), represents the toxic load exponent. In our case the slope = −1.17, thus, the toxic load exponent is 1.17, a value >1. Continuing to estimate phosgene toxicity with a linear C x t assumption over-predicts the exposure duration to reach lethality, hence, under-predicting risk in an acute exposure scenario; exactly what we encountered in our initial study with phosgene concentrations ranging from 191 to 316 mg/m^3 over 10 min (Table 1, Study 1). In a real-

world situation, the lethality is compounded by phosgene's greater atmospheric density, resulting in its slow dissipation from the point of release, and its mild odor, which does not generate immediate alarm in exposure victims. It should be noted that non-linearity can also apply to exposure duration and a power exponent can be determined for the time variable in Haber's rule (Belkebir et al. 2011). An analysis of exposure duration for 21 chemicals found none had an exponent = 1 (i.e. C x t^1) causing the researchers to recommend caution in risk assessments using Haber's rule (Belkebir et al. 2011).

For FDA licensure of a treatment for phosgene exposure, small and large animal models are often necessary when human trials are not available (Chemical Warfare Toxicology: Volume 2: Management of Poisoning 2018). Earlier phosgene research has involved the use of dogs, rabbits, rats, mice and guinea pigs resulting in an assortment of, and sometimes contradictory, LC_{50} values (see Table 25.3 in Bast and Glass-Mattie 2015). Since there was no definitive rat species model for a 10 min exposure to phosgene that we could rely on for our future therapeutic development studies, the small animal model we chose is the Wistar rat. It was selected because the animals respond in a similar manner to phosgene as compared to humans over a 24-h post-exposure latent injury phase, due to phosgene toxicity. Two earlier studies had made strides investigating phosgene exposure for various durations, including 10 min, in rats (Zwart et al. 1990; Pauluhn 2006a). A more robust 10 min exposure model had to be developed to address the problem of acute phosgene exposure. We incorporated greater accuracy intoxicating rats with a nose-only system, greater precision in the measurement of phosgene delivered to that system by using FTIR spectrometry, more rapid equilibration of the phosgene concentrations, and we acclimated the rats for several days to the stress of confinement in the system presumably removing confounding stress responses in the animal.

Our physiological observations in rats corroborate observations in other animal models. Studies in rabbits have demonstrated that phosgene induced pulmonary edema is caused by increased capillary permeability and not due to any alteration in atrial pressure or other hemodynamic factors (Russell et al. 2006). BALF analysis comparing control and phosgene exposed rats has shown marked changes in inflammatory (Chen et al. 2011) and cytokine mediators (Sciuto, Clapp, et al. 2003), as well as antioxidant enzymes (Sciuto, Cascio, et al. 2003). These processes appear to be driven by the phosphorylation of the JNK/SAPK and p38 MAPK pathways, in turn upregulating i) the expression of cyclooxygenase2 (COX-2) which leads to prostaglandin E_2 production and inflammation, and ii) the expression of inducible nitric oxide synthase (iNOS) which leads to oxidative stress (Chen et al. 2011). Our results confirm pulmonary edema is caused by increased capillary permeability in the rat: both macroscopically (Figure 4) and microscopically (Figure 5A) the leakage of blood cells into the pulmonary interstitium with increased phosgene exposure, particularly at dosages previously assessed as non-lethal (Pauluhn 2006a). Furthermore, it was observed that airway exfoliation and edema constituted primary lesions; and the presence of fibrin strands, even under less toxic exposures, is a physiological reaction of the body to phosgene (Figure 5B). We have quantified this observation by determining that a lung / body weight ratio ≤ 0.02 correlates with survival and that ratios ≥ 0.02 are significantly prevalent (Table 2) above our calculated LC_{50} determination of 129.2 mg/m^3 (31.5 ppm). We have verified this LC_{50} by intoxicating another group of nine rats and determining that the survival and

lung / body weight ratios (Figure 6) are no different from an earlier cohort of 5 rats exposed close to the LC_{50} (134.6 mg/m^3). Unlike previous LC_{50} determinations for a 10 min exposure (Zwart et al. 1990; Pauluhn 2006a; Bast and Glass-Mattie 2015), our LCt_{50} of 1292 mg/m^3 × min corresponds to the expected range of 1,000 – 2,000 mg/m^3 × min for phosgene in most experimental animal species as determined by the World Health Organization (Public Health England 2016). Our value of 1292 mg/m^3 × min also suggests greater toxicity with a 10 min exposure in rats compared to the accepted LCt_{50} in humans of ~2000 mg/m^3 × min (Public Health England 2016). Therefore, a 10-min nasal exposure of phosgene in a 24-h rat survival model is anticipated to be an acceptable small animal model for real-world phosgene exposures like those expected from a terrorist attack, battlefield deployment, or industrial accident.

Conclusions

The toxicity of phosgene delivered by nose-only inhalation to Wistar rats for 10 min in our model system is significantly higher than previously reported. Data reported herein indicate that we achieved a robust model and, in the process, demonstrated that phosgene does not adhere to a linear interpretation of Haber's rule at short duration, high concentration exposures. Using this system, the LC_{50} for a 10-min exposure of phosgene is 129.2 mg/m^3. When aligned with the earlier reported LC_{50} results at extended times (Pauluhn 2006a), these data indicate that phosgene departs from a strict linear adherence to Haber's rule and conforms to a toxic load model for phosgene. The toxic load exponent is 1.17, indicating that high concentration exposures of short duration are more toxic than low concentration exposures of long duration. Considering this characteristic, phosgene appears to have commonality with sarin and cyanide as reported by others (Spruit et al. 2000; Mioduszewski et al. 2002; Pauluhn 2006a; Sweeney et al. 2015). We suggest our model system is not only more accurate in the verification of phosgene delivered to the animals but also is more reflective of relevant real-world scenarios, whereby, phosgene exposure will be of a short-duration and at a high concentration whether it is used as a chemical warfare agent, a terrorist action, or released during an industrial accident.

The model is immediately applicable for future studies on the toxicology of phosgene and in the development of effective therapeutics, in which these observations and the toxic load value, are crucial to accurately determine the efficacy of novel treatment protocols. We believe new treatment strategies are necessary given the current questionable standard of care, which is relegated to providing methylprednisolone (Smith et al. 2009), supplemental oxygen (Grainge, Jugg, et al. 2010) and telling the victim to not physically exert themselves (Russell et al. 2006). Using the exposure levels investigated here, efforts are under way to determine the utility of intracellular targeting of Hsp72 for pulmonary cytoprotection following phosgene intoxication (Parseghian et al. 2016).

Acknowledgements

These studies were supported by the National Institute of Environmental Health Sciences (NIEHS) of the National Institutes of Health (NIH) under Award Number R21ES024028. The content is solely the responsibility of the authors and does not necessarily represent the official views of the NIH. The authors wish to thank Dr. Claire Croutch, Ed Sistrunk, Karin Bauer and Dr. Tom Carpenter for excellent technical assistance on this project.

References

Baggett RK, Simpkins BK. 2018 Chapter 6, Critical Infrastructure Threats and Hazards Homeland Security and Critical Infrastructure Protection. 2 ed. Santa Barbara, Denver: Praeger, ABC-CLIO, LLC; p. 146–170.

Bast CB, Glass-Mattie DF. 2015 Chapter 25, Phosgene In: Gupta RC, editor. Handbook of Toxicology of Chemical Warfare Agents. 2 ed. New York, San Francisco, London: Academic Press, Inc.; p. 327–335.

Belkebir E, Rousselle C, Duboudin C, Bodin L, Bonvallot N. 2011 Haber's rule duration adjustments should not be used systematically for risk assessment in public health decision-making. Toxicol Lett. 204(2-3):148–155. [PubMed: 21565258]

Borak J, Diller WF. 2001 Phosgene exposure: mechanisms of injury and treatment strategies. J Occup Environ Med. 43(2):110–119. [PubMed: 11227628]

Chemical Warfare Toxicology: Volume 2: Management of Poisoning. 2018 Worek F, Jenner J, Thiermann H, editors. London: Royal Society of Chemistry.

Chen HL, Bai H, Xi MM, Liu R, Qin XJ, Liang X, Zhang W, Zhang XD, Li WL, Hai CX. 2011 Ethyl pyruvate protects rats from phosgene-induced pulmonary edema by inhibiting cyclooxygenase2 and inducible nitric oxide synthase expression. J Appl Toxicol. 33(1):71–77. [PubMed: 21818760]

Diller WF. 1985 Pathogenesis of phosgene poisoning. Toxicol Ind Health. 1(2):7–15.

Fentener van Vlissingen JM, Borrens M, Girod A, Lelovas P, Morrison F, Torres YS. 2015 The reporting of clinical signs in laboratory animals: FELASA Working Group Report. Lab Anim. 49(4):267–283. [PubMed: 25957286]

Filipczak PT, Senft AP, Seagrave J, Weber W, Kuehl PJ, Fredenburgh LE, McDonald JD, Baron RM. 2015 NOS-2 Inhibition in Phosgene-Induced Acute Lung Injury. Toxicol Sci. 146(1):89–100. [PubMed: 25870319]

Finney DJ. 1952 Probit Analysis. 2 ed. New York: Cambridge University Press.

Ghio AJ, Kennedy TP, Hatch GE, Tepper JS. 1991 Reduction of neutrophil influx diminishes lung injury and mortality following phosgene inhalation. J Appl Physiol. 71(2):657–665. [PubMed: 1657861]

Grainge C, Brown R, Jugg BJ, Smith AJ, Mann TM, Jenner J, Rice P, Parkhouse DA. 2009 Early treatment with nebulised salbutamol worsens physiological measures and does not improve survival following phosgene induced acute lung injury. J R Army Med Corps. 155(2):105–109. [PubMed: 20095175]

Grainge C, Jugg BJ, Smith AJ, Brown RF, Jenner J, Parkhouse DA, Rice P. 2010 Delayed low-dose supplemental oxygen improves survival following phosgene-induced acute lung injury. Inhal Toxicol. 22(7):552–560. [PubMed: 20384554]

Grainge C, Rice P. 2010 Management of phosgene-induced acute lung injury. Clin Toxicol (Phila). 48(6):497–508. [PubMed: 20849339]

Grainge C, Smith AJ, Jugg BJ, Fairhall SJ, Mann T, Perrott R, Jenner J, Millar T, Rice P. 2010 Furosemide in the treatment of phosgene induced acute lung injury. J R Army Med Corps. 156(4): 245–250. [PubMed: 21275359]

Haber F 1924 Zur geschichte des gaskrieges (On the history of gas warfare) In Fünf Vorträge aus den Jahren 1920-1923 (Five Lectures from the Years 1920-1923). Springer, Berlin pp 76–92.

He DK, Shao YR, Zhang L, Shen J, Zhong ZY, Wang J, Xu G. 2014 Adenovirus-delivered angiopoietin-1 suppresses NF-kappaB and p38 MAPK and attenuates inflammatory responses in phosgene-induced acute lung injury. Inhal Toxicol. 26(3):185–192. [PubMed: 24517841]

Krishna G, Goel S. 2015 Chapter 45, Alternative animal toxicity testing of chemical warfare agents In: Gupta RC, editor. Handbook of Toxicology of Chemical Warfare Agents. 2 ed. New York, San Francisco, London: Academic Press, Inc.; p. 657–673.

Li W, Pauluhn J. 2014 Chapter 19, Mechanisms involved in the inhalation toxicity of phosgeneIn: Salem H, Katz SA, editors. Inhalation Toxicology. 3 ed. Boca Raton, Florida: CRC Press; p. 459–483.

Miller FJ, Schlosser PM, Janszen DB. 2000 Haber's rule: a special case in a family of curves relating concentration and duration of exposure to a fixed level of response for a given endpoint. Toxicology. 149(1):21–34. [PubMed: 10963858]

Mioduszewski R, Manthei J, Way R, Burnett D, Gaviola B, Muse W, Thomson S, Sommerville D, Crosier R. 2002 Interaction of exposure concentration and duration in determining acute toxic effects of sarin vapor in rats. Toxicol Sci. 66(2):176–184. [PubMed: 11896284]

Narciso SP, Nadziejko E, Chen LC, Gordon T, Nadziejko C. 2003 Adaptation to stress induced by restraining rats and mice in nose-only inhalation holders. Inhal Toxicol. 15(11):1133–1143. [PubMed: 12955618]

Parker JC, Townsley MI. 2004 Evaluation of lung injury in rats and mice. Am J Physiol Lung Cell Mol Physiol. 286(2):L231–L246. [PubMed: 14711798]

Parseghian MH, Hobson ST, Richieri RA. 2016 Targeted heat shock protein 72 for pulmonary cytoprotection. Ann N Y Acad Sci. 1374(1):78–85. [PubMed: 27152638]

Pauluhn J 2006a Acute nose-only exposure of rats to phosgene. Part I: concentration × time dependence of LC50s, nonlethal-threshold concentrations, and analysis of breathing patterns. Inhal Toxicol. 18(6):423–435. [PubMed: 16556582]

Pauluhn J 2006b Acute nose-only exposure of rats to phosgene. Part II. Concentration × time dependence of changes in bronchoalveolar lavage during a follow-up period of 3 months. Inhal Toxicol. 18(9):595–607. [PubMed: 16864551]

Public Health England. 2016 Phosgene: Toxicological overview. Report Number 2014790.

Rozman KK, Doull J. 2000 Dose and time as variables of toxicity. Toxicology. 144(1-3):169–178. [PubMed: 10781885]

Russell D, Blaine PG, Rice P. 2006 Clinical management of casualties exposed to lung damaging agents: a critical review. Emerg Med J. 23:421–424. [PubMed: 16714497]

Sciuto AM, Cascio MB, Moran TS, Forster JS. 2003 The fate of antioxidant enzymes in bronchoalveolar lavage fluid over 7 days in mice with acute lung injury. Inhal Toxicol. 15(7):675–685. [PubMed: 12754689]

Sciuto AM, Clapp DL, Hess ZA, Moran TS. 2003 The temporal profile of cytokines in the bronchoalveolar lavage fluid in mice exposed to the industrial gas phosgene. Inhal Toxicol. 15(7): 687–700. [PubMed: 12754690]

Sciuto AM, Hurt HH. 2004 Therapeutic treatments of phosgene-induced lung injury. Inhal Toxicol. 16(8):565–580. [PubMed: 15204747]

Sciuto AM, Stotts RR, Hurt HH. 1996 Efficacy of ibuprofen and pentoxifylline in the treatment of phosgene-induced acute lung injury. J Appl Toxicol. 16(5):381–384. [PubMed: 8889788]

Shen J, Gan Z, Zhao J, Zhang L, Xu G. 2014 Ulinastatin reduces pathogenesis of phosgene-induced acute lung injury in rats. Toxicol Ind Health. 30(9):785–793. [PubMed: 23075575]

Smith A, Brown R, Jugg B, Platt J, Mann T, Masey C, Jenner J, Rice P. 2009 The effect of steroid treatment with inhaled budesonide or intravenous methylprednisolone on phosgene-induced acute lung injury in a porcine model. Mil Med. 174(12):1287–1294. [PubMed: 20055070]

Spruit HE, Langenberg JP, Trap HC, van der Wiel HJ, Helmich RB, van Helden HP, Benschop HP. 2000 Intravenous and inhalation toxicokinetics of sarin stereoisomers in atropinized guinea pigs. Toxicol Appl Pharmacol. 169(3):249–254. [PubMed: 11133347]

Summerhill EM, Hoyle GW, Jordt SE, Jugg BJ, Martin JG, Matalon S, Patterson SE, Prezant DJ, Sciuto AM, Svendsen ER et al. 2017 An Official American Thoracic Society Workshop Report: Chemical Inhalational Disasters. Biology of Lung Injury, Development of Novel Therapeutics, and Medical Preparedness. Ann Am Thorac Soc. 14(6):1060–1072. [PubMed: 28418689]

Sweeney LM, Sommerville DR, Channel SR, Sharits BC, Gargas NM, Gut CP Jr. 2015 Evaluating the validity and applicable domain of the toxic load model: impact of concentration vs. time profile on inhalation lethality of hydrogen cyanide. Regul Toxicol Pharmacol. 71(3):571–584. [PubMed: 25720732]

ten Berge WF, Zwart A, Appelman LM. 1986 Concentration-time mortality response relationship of irritant and systemically acting vapours and gases. J Hazard Mater. 13:301–309.

Warren E 1900 On the reaction of *Daphnia magna* (Straus) to certain changes in its environment. Q. J. Microsc. Sci. 43:199–224.

Weinrich SL, Lawrence AE, Burr JK, Grotte JH, Laviolet LL. 2008 A comparative analysis of toxicity models. Alexandria, Virginia. IDA Paper P-4327.

Zwart A, Arts JHE, Klokman-Houweling JM, Schoen ED. 1990 Determination of Concentration-Time-Mortality Relationships to Replace LC50 Values. Inhal Toxicol. 2(2):105–117.

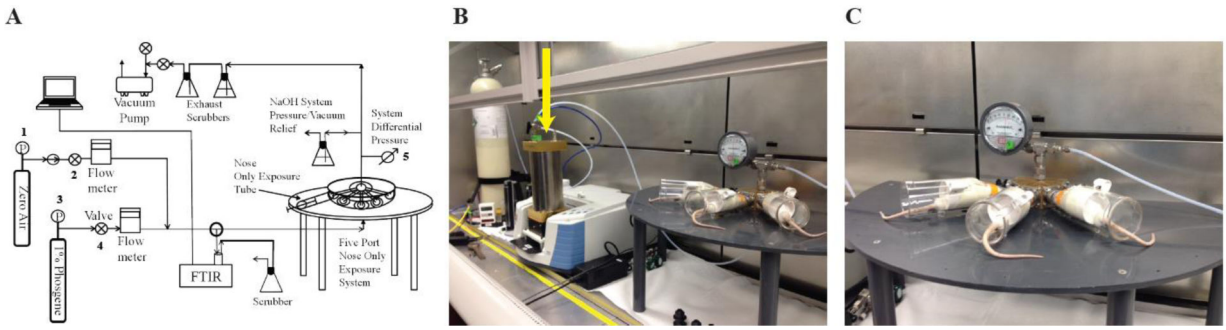

Figure 1. Phosgene Nose-Only Exposure System.

A) Schematic representation of the system set up. Phosgene delivered to the rats is adjusted by valves on the compressed air tank (1) and in line (2) prior to mixing, as well as valves on the phosgene tank (3) and in line (4) prior to mixing. Flow was monitored with two in-line mass flow meters and accuracy of the delivered concentration verified by FTIR spectrometry. Atmospheric pressure was maintained within the system by using a gauge (5) to monitor the differential pressure, which was kept at zero, between the gas tanks and the outlet ports. B) System assembled within a hood. The FTIR spectrometer is denoted by the yellow arrow. C) Rats being acclimated to the exposure tubes.

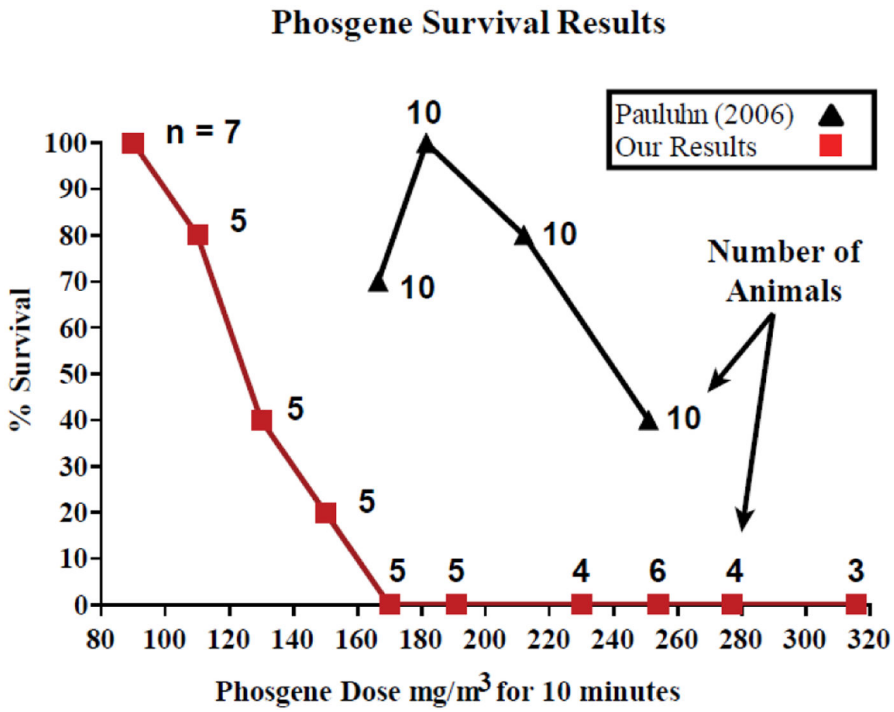

Figure 2. Comparison of Survival Results.

The study results here cover a broader range of phosgene concentrations than the earlier study by Pauluhn (2006a). The lower survival curve with our study aligns with the steep toxicity profile seen at higher exposures of 30, 60 and 240 min. The number of rats per exposure group are shown above each data point.

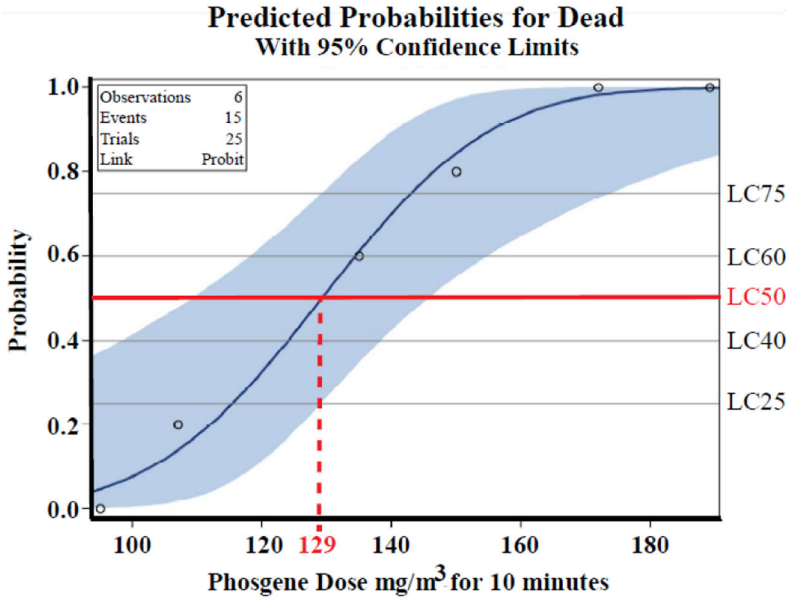

Figure 3. Probit analysis.

A linear regression analysis of the dose response curve has determined the likely LC_{50} for a 10 min exposure of phosgene is 129.2 mg/m^3(31.5 ppm). The actual concentrations achieved (see Table 1) are plotted as open circles (○) and the 95% confidence limits are shaded in gray (published journal) or in blue (online journal).

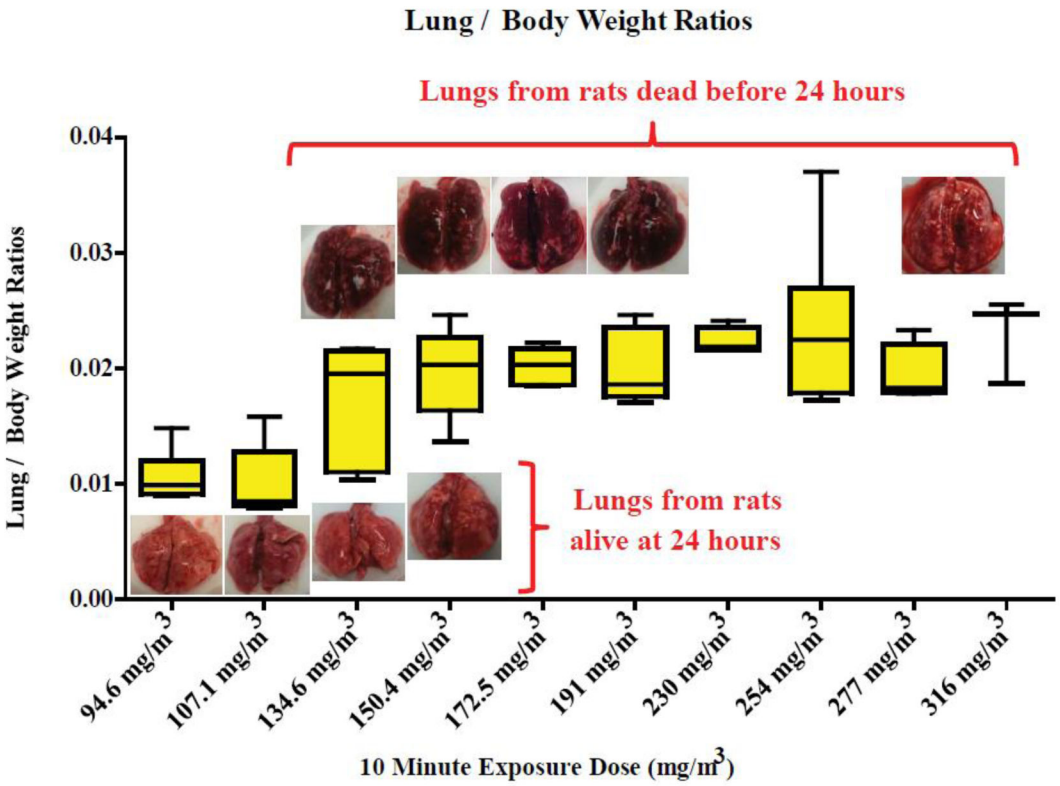

Figure 4. Box plot of Lung / Body Weight ratios.

An indirect measure of edema and extravasated protein in all rats at the end of the 24 h study. Lungs with greater amounts of edema correlate with the higher phosgene concentrations. Rats that expired prior to the 24-h timepoint show greater amounts of hemorrhaging, giving the tissue a burgundy coloration. The number of rats in each targeted exposure is listed in Figure 2 and in Table 1.

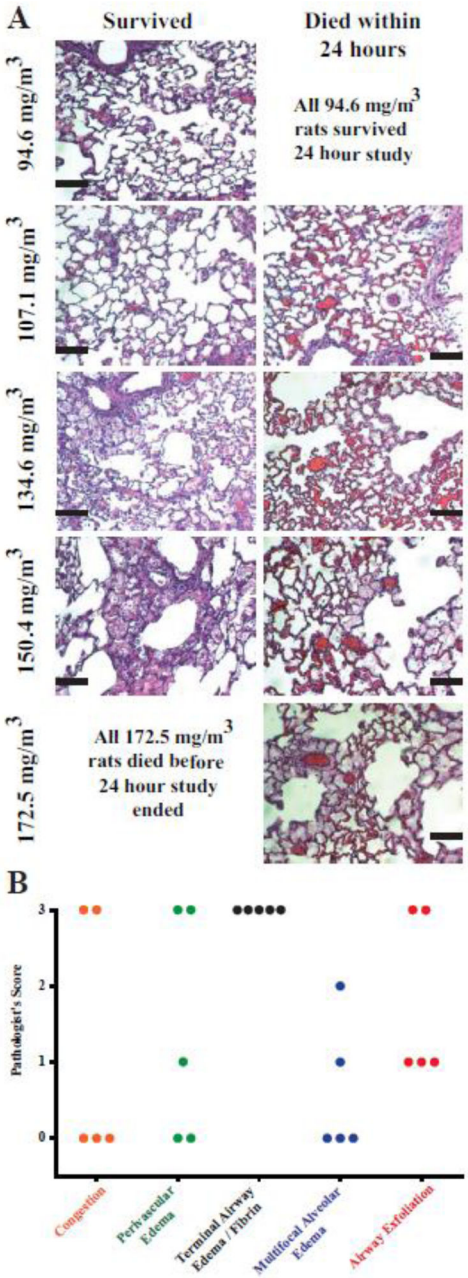

Figure 5. Pathology of lung tissues.

A) H&E staining of lung sections reveals hemorrhaging at the microscopic level with greater prevalence in the higher dosages. Images captured at 150X using a 42 msec exposure. Scale Bar = 100 ¼m. B) Scoring of other lesions for the 134.6 mg/m^3 rats (n=5):

Congestion: 0 = no congestion; 1 = mild patchy; 2 = moderate coalescing; 3 = diffuse congestion.

Perivascular Edema: 0 = no perivascular edema; 1 = patchy, mild; 2 = moderate multifocal; 3 = severe, all.

Terminal Airway Edema / Fibrin: 0 = no terminal airway fibrin; 1 = minimal, isolated; 2 = multifocal, limited; 3 = disseminated, moderate.

Multifocal Alveolar Edema: 0 = none; 1 = multifocal, mild; 2 = multifocal, moderate; 3 = multifocal, coalescing, moderate to severe.

Airway Exfoliation: 1=patchy degeneration, still attached; 2 = Extensive degeneration, moderate blebbing, mild exfoliation; 3 = Extensive detachment to complete exfoliation, generally severe degeneration or frank necrosis of the epithelial cells.

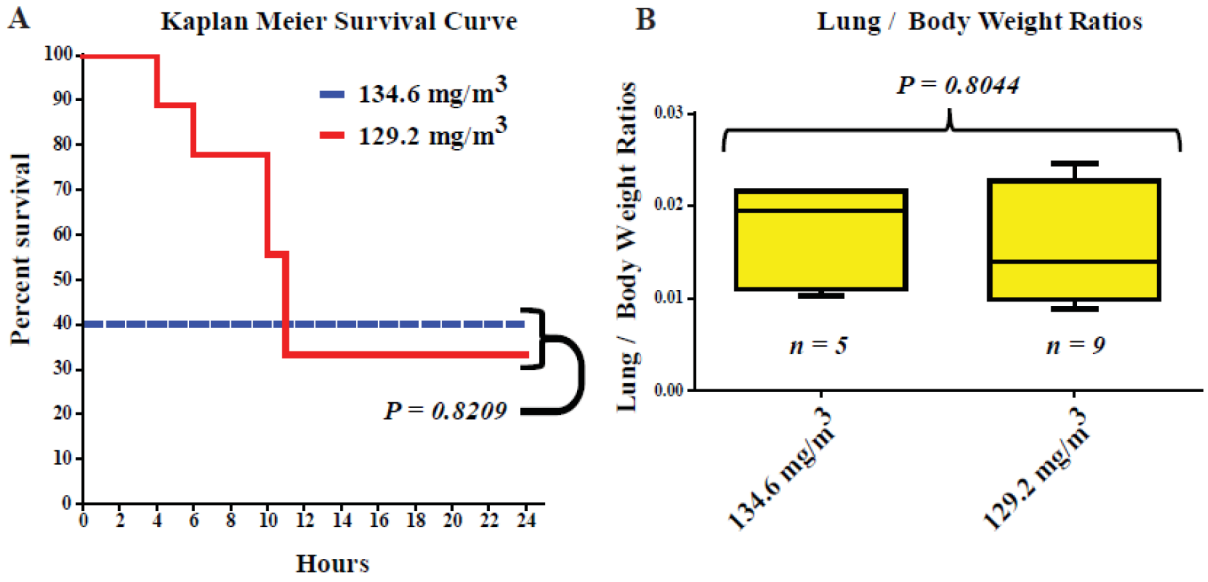

Figure 6. 24 h Survival Study

A) Kaplan-Meier survival curve for rats receiving 129.2 mg/m^3 phosgene for 10 min (red line; n = 9 rats) compared to the 40% survival seen at 24 hours of rats exposed to 134.6 mg/m^3 phosgene for 10 min (blue line; n = 5 rats). B) Lung / Body Weight ratios for all nine rats exposed to 129.2 mg/m^3 in the study compared to the 134.6 mg/m^3 phosgene results presented in Figure 4.

A

Time (minutes)	LC_{50} (mg/m^3)	LCt_{50} (mg/m^3-min)
10	129.2	1292
30	54.5	1635
60	31.3	1878
240	8.6	2064

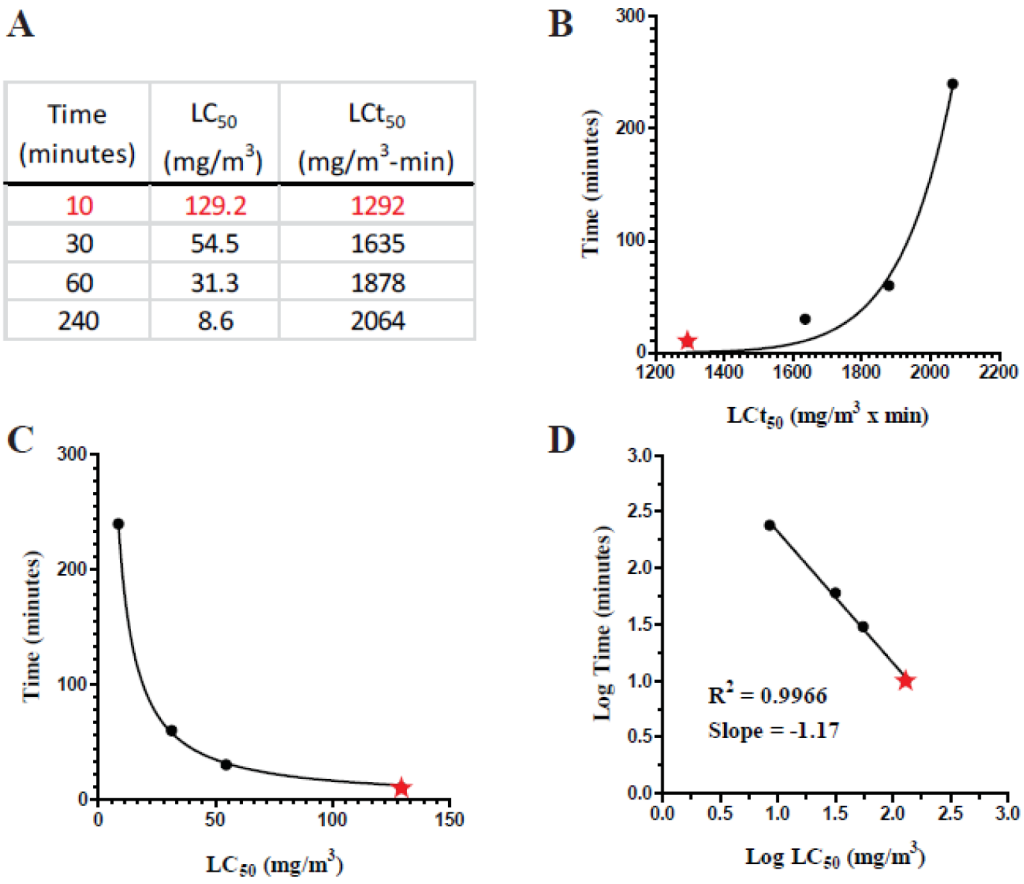

Figure 7. Determining the Toxic Load Exponent

A) Table of LC_{50} and LCt_{50} results determined from our 10 min exposure studies and Pauluhn's 30, 60 and 240 min studies. B) Plotting the LCt_{50} versus exposure times reveals a deviation in the lethal dosages from Haber's rule. C) Survival in the form of the LC_{50} parameter was used to determine the toxic load exponent for phosgene. D) Plotting LC_{50} versus exposure times on a logarithmic scale allows for a regressive determination of n_e as 1.17. The LC_{50} values for 30, 60 and 240 min were obtained from Pauluhn (2006a) and are denoted by (•) data points, while the 10 min LC_{50} value obtained in this study is denoted by a star (★).

Table 1

Flow rates used to achieve targeted phosgene concentrations

Experiment	# of Rats	Phosgene Flow Rate (cc / minute)	Air Dilution Flow Rate (L / minute)	Phosgene Exposure Achieved according to FTIR (mg / m^3)
Study 1	3	39	5	316
	4	39	5.7	277
	6	39	6.2	254
	4	39	6.9	230
	3*	39	8.3	191
Study 2	2*	23.5	5	189.6
	5	23.5	5.6	172.5
	5	23.5	6.3	150.4
	5	23.5	7.3	134.6
	5	23.5	8.6	107.1
	7	23.5	10.5	94.6

* Data from these five rats are treated as a single cohort (191 mg/m^3) in plotting Figures 2, 3 and 4.

Table 2

Lung / Mass Ratios with Unpaired T-Test Comparison of Cohorts to the 94.6 mg/m^3 exposure.

mg/m^3	**94.6**	**107.1**	**134.6**	**150.4**	**172.5**	**191**	**230**	**254**	**277**	**316**
Lung / Body Weight Ratios	0.0093	0.0085	0.0117	0.0207	0.0186	0.0170	0.0241	0.0235	0.0233	0.0255
	0.0120	0.0083	0.0214	0.0137	0.0185	0.0181	0.0216	0.0236	0.0178	0.0247
	0.0091	0.0158	0.0217	0.0246	0.0212	0.0246	0.0220	0.0181	0.0184	0.0187
	0.0090	0.0080	0.0195	0.0190	0.0203	0.0186	0.0217	0.0370	0.0182	
	0.0099	0.0097	0.0103	0.0203	0.0222	0.0224		0.0214		
	0.0148							0.0172		
	0.0099									
P values ***Significance***	***N/A***	***0.7480***	***0.0181*** *	***0.0004*** ***	***<0.0001*** ***	***<0.0001*** ***	**<0.0001** ***	***0.0008*** ***	***0.0002*** ***	***0.0001*** ***