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Comprehensive Review

Inhalation toxicity of cyclic semi-volatile methylsiloxanes: Disentangling the conundrum of phase-specific adaptations from adverse outcomes

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ABSTRACT

This paper compares the phase-specific inhalation toxicity of the cyclic semi-volatile methylsiloxanes (cVMSs) D4, D5 and D6. The objectives of this paper are to re-analyze information from acute to chronic inhalation studies on rats with these cVMSs to identify the unifying principles of phase-specific toxicity at the portal-of-entry and if they depend on acute, acute-on-chronic or chronic mechanisms. This re-analysis supports the hypothesis that concentrations must be high enough to exceed the vapor saturation at any given temperature for stabilizing the aerosol phase and evoking phase-specific effects at sites of the respiratory tract susceptible to the cVMSs-specific physicochemical properties amphiphilicity and surface tension. In summary, the portal-of-entry effects and related findings appear to be acute in nature and specific to liquid aerosol. The repeated inhalation exposure studies with D4 and D5 up to two years in duration did not reveal chronic aggravations of portal of entry outcomes. Findings at a pulmonary location where amphiphilic surfactant molecules are present appear to be caused by the acute adaptation to deposited dose. Such outcome should better be described as a high-dose liquid aerosol phenomenon imparted by the physicochemical properties "liquid" and "hydrophobic". This calls for a phase-specific human risk characterization of cVMSs.

1. Introduction

This paper compares the phase-specific inhalation toxicity of the cyclic semi-volatile octamethylcyclotetrasiloxane (D4; CAS RN 556-67-2), decamethylcyclopentasiloxane (D5; CAS RN 541-02-6), and dodecamethylcyclohexasiloxane (D6; CAS RN 540-97-6). These cyclic methylsiloxanes are used as intermediates in the production of silicon-based polymers for numerous industrial and consumer applications. As this paper focuses on inhalation toxicology-based risk characterization and assessment, the most critical exposure limiting mode of toxic action needs to be identified for defining any toxic outcome-specific metric of exposure. For a volatile substance, the commonly applied metric of choice is the molar volume 'ppm' (1 part per million). However, under the given conditions of inhalation studies, the generation of test atmospheres requires evaporation of the liquid phase well above the vapor saturation pressure (Vsat) resulting in dynamic mixed phase vapor-liquid equilibria difficult to quantify phase-specifically. Such conditions mandate the 'total mass concentration' as lead metric as called for by OECD testing guidelines (OECD, 2018). This pragmatic approach is challenged if any phase-specific portal-of-entry related toxicity occurs because the most critical phase must guide human risk assessment. The mixture of phases existent under the highly contrived conditions in inhalation chambers exceeding Vsat can hardly be reproduced under real-life exposure conditions where passive evaporation of the liquid prevails. This explains why inhalation toxicologists would classify a compound present in a vapor-liquid equilibrium to be semi-volatile, whereas environmental toxicologists focusing on indoor environments would prefer the term volatile. The upper respiratory tract sensory irritation (pungency) potency (RD_{50}) is linked to the Vsat, i.e., the lower the Vsat, the more potent becomes pungency (and associated species-specific nociceptive responses) (Mølhave and Nielsen, 1992). The objective of this analysis is to compare the interrelationship of the physicochemical property 'Vsat' and likelihood of presence of aerosol with the portal-of-entry outcomes from short-to long-term inhalation studies. As a class sharing the same toxicological properties, the three homologues selected for this comparison of *cyclic volatile methyl siloxanes* are abbreviated 'cVMS'.

Their molecular weights range from 300 (D4) to 445 (D6) and are determined by the number of $[\text{-}(CH_3)_2Si\text{–}O\text{-}]_n$ moieties forming a cyclic backbone structure from n = 4 to 6. The vapor saturation pressures (V_{sat})

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prevailing at ≈25 °C are ≈15,000, ≈2,500, and ≈400 mg/m^3 for D4, D5, and D6, respectively (Fig. 1). Their respective aqueous solubilities at 23 °C are 189.9, 46.0, and 11.6 mmol/L (Varaprath et al., 1996). A comprehensive overview of the physicochemical properties of these cVMSs is detailed elsewhere (DK-EPA, 2014; ECHA, 2020; SCCS, 2010, 2016). Unique physicochemical properties of liquid cVMSs are manifested by their adhesion strength, surface spreading, and amphiphilicity caused by their chemically inert liquid interface and lack of reactivity (Mojsiewicz-Pieńkowska et al., 2016). These liquid-specific physicochemical properties require a phase-specific approach for hazard identification and risk characterization. While liquid aerosols of cVMSs will be deposited onto surfaces of the respiratory tract following the principles of aerodynamics, the vapor phase follows the principles of diffusion and partitioning. These characteristics of interactions require a prudent dual approach in translating toxicological data from inhalation studies on animals to humans (Kuempel et al., 2017; US-EPA, 2004). Taken as a whole, the physicochemical properties conveyed by the liquid aerosol phase may trigger adaptive responses and require concentrations equal and above the vapor pressure of cVMSs. Although such conditions can hardly be attained under the normal practices of use, potential pitfalls related to the generation and characterization of exposure atmospheres, modes of inhalation exposure, and artifacts potentially affecting the outcome must be considered as well.

Inhalation studies present significant methodological challenges for semi-volatile cVMS. Under conditions used for toxicity testing by the inhalation route, these substances may be present as vapor at lower concentrations but attain a dynamic thermodynamic equilibrium of the vapor phase and liquid aerosol phase at and above to V_{sat}. This issue can be further complicated depending on the type of generator and gradients in temperatures and dilutions applied. Depending on these variables and gradients, each generation and exposure system may have its own characteristic of aerosol coagulation and growth in relation to the concentration of condensable vapors. The average residence time of aerosol within small nose-only and larger whole-body chambers may differ appreciably, resulting in generation- and mode of exposure-specific artifacts. Consequently, atmospheres assumed to be free of aerosol below V_{sat} may still contain appreciable amounts of this phase under specific conditions. The extreme dynamics of these concatenated events complicate the quantification of liquid aerosol in inhalation chamber atmospheres.

The standard Draize eye irritation test with 100 μl of neat D4 or D5 liquid instilled into the conjunctival sac of rabbits evoked hyperemic vessels in the conjunctivae (D4) or no response (D5) (SCCS, 2010). These and related data do not qualify these compounds to be classified as *inflammatory irritants* but could not exclude sensory irritation above Vsat (Mølhave and Nielsen, 1992). If inhaled as mixed liquid aerosol in a vapor saturated atmosphere each phase interacts differently with the various tissues of the respiratory tract. Physicochemical interactions may mimic inhaled dose-proportionally on the protective systems of the respiratory tract to maintain its functional integrity, e.g. increase production and discharge of mucous and pulmonary surfactant followed by adaptive increase of phagocytic cells (alveolar macrophages) to compensate for the increased needs to keep the lung free of foreign material. The adaptive increase to high dose is an essential requirement for the general well-being of an animal (including humans). However, such constitutive responses to high-dose challenges are often perceived as an insult suggesting that adversity has occurred (Lewis et al., 2002; Palazzi et al., 2016; Pandiri et al., 2017). The approach taken in this re-analysis is to stratify findings according to phase-specific outcomes and No-Observed Adverse-Effect Levels (NOAELs).

An additional dimension of complexity needs to be addressed when assessing inhalation studies for phase-specific outcomes and their significance to human health. For example, high-concentration mixed-phase phenomena may stimulate the nociceptive sensory system of the rat by liquid phase-specific physical factors (Pauluhn, 2018a). These physical factors may also cause acute interferences in lung physiology

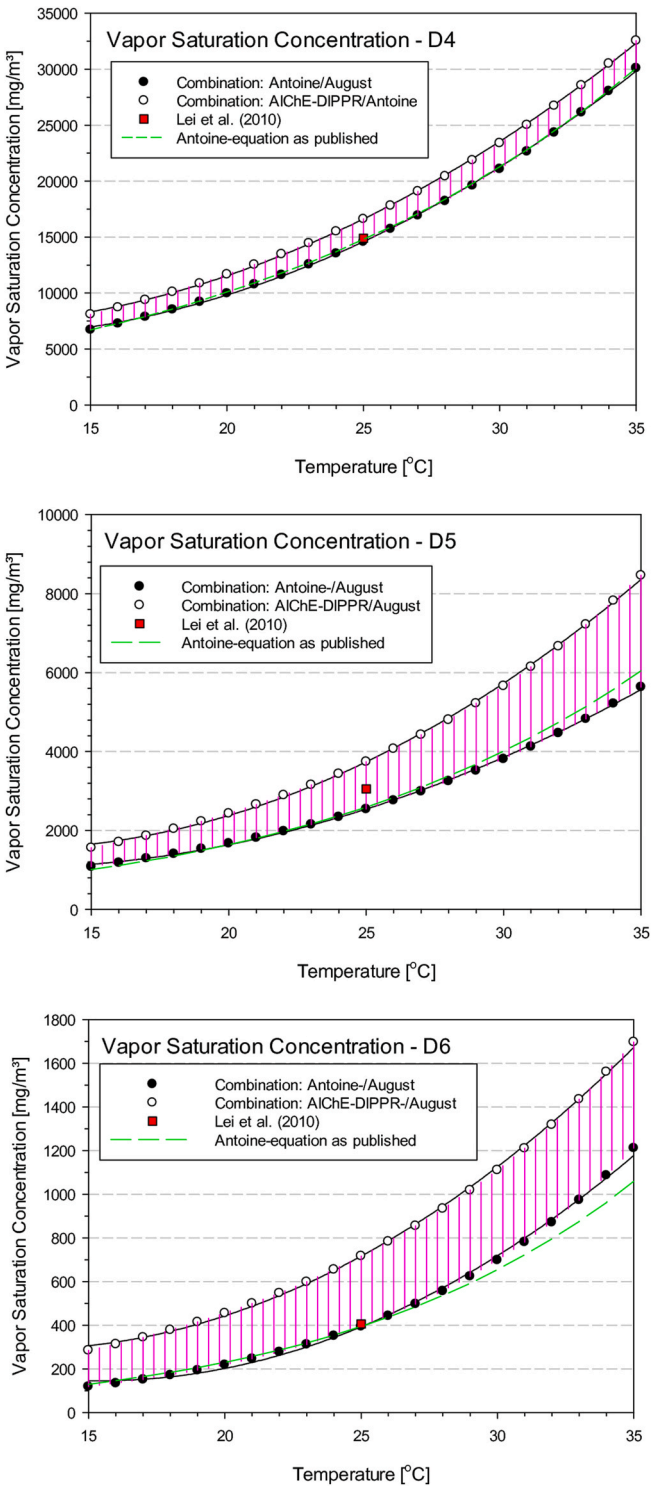

Fig. 1. The pairs of published vapor pressures at 20 and 25 °C from Brooke et al. (2009a-c) using the Antoine's (filled circles) and the AIChE DIPPR (open circles) method were re-calculated by the August's method (Fortran source code programmed by the author of this appraisal). The area between the curves (fill pattern vertical magenta lines) are intended to represent the "thermodynamically plausible" range; however, upon cross-validation using the vapor pressures at 25 °C published by Lei et al. (2010), Antoine's equation-based data appear to provide the best match. Calculations using August's equation were supported by using the original Antoine method (dashed line) as parameterized by Brooke at al. (2009a-c).

controlled by Starling's and LaPlace's Laws (Rhoades and Bell, 2013). While there is some descriptive material later in this paper that provides information that these laws have implications for the respiratory toxicity of cVMS inhaled as liquid aerosol, the following concise overview summarizes the mechanistic background. To be exact, the term Starling's law is used for the Starling-Landis equation which interrelates the role of hydrostatic pressure (measured as pulmonary capillary wedge pressure, PCWP) and colloid osmotic pressure (COP) in determining fluid movement across the pulmonary capillaries. Vascular fluid is filtered to the interstitial space under a hydrostatic pressure gradient (push) which is opposed by a capillary COP. The Starling dependent filtration to the septal interstitium is balanced by lymphatic drainage which reflects a complex balance between at least three sets of forces: 1) those governing microvascular-interstitial fluid exchanges; 2) elastic lung recoil forces; and 3) alveolar surface forces at the air–tissue interface covered by surfactant generating the negative absorption pressure of the lymphatic pump and the pressure in the extravascular compartment, respectively. When lymphatic flow cannot match the increase in filtration rate fluid drainage becomes overwhelmed. The alveolar lining is coated with a special surface-active agent, pulmonary surfactant, which not only lowers surface tension at the gas–liquid interface but also changes surface tension with changes in alveolar diameter. The pressure developed in an alveolus is given by the LaPlace law. The surface tension of pulmonary surfactant has important ramifications for maintaining alveolar pressure gradients and stability. In a sphere such as an alveolus, surface tension produces a force that pulls inwardly and creates an internal pressure preventing alveolar collapse (atelectasis). The adjacent alveoli become overdistended when atelectasis occurs. Atelectatic alveoli are still perfused but not ventilated which triggers the hypoxic pulmonary vasoconstriction (HPV) reflex that mitigates hypoxemia by minimizing venous admixture. In the context of this paper, this concise overview of basic pulmonary physiology delineates that the liquid phase of lipophilic substances inhaled at extremely high dose-rates may "titrate" pulmonary surfactant to a level leading to increased interstitial fluid and related increased lymphatic clearance. Hence, from any mechanistic perspective, the most sensitive endpoint characterizing any surfactant-related pulmonary fluid imbalance is integrated by the corresponding increased absolute lung weight.

The lung has multiple compensatory adaptive mechanisms in place to counteract and prevent such acute phenomena to occur. Acutely irritant and non-irritant etiopathologies can readily be distinguished by 'acute-on-chronic' aggravations, an artifact often observed for lung irritants in repeated inhalation studies. The 'acute-on-chronic' aggravation typically occurs when the acute response is inflammation-related. Any re-exposure at irritant levels causes aggravation and not, as often surmised, accumulation. To the contrary, the non-irritant related adaptive response, after attaining its homeostatic steady state, remains stable, i.e., the acute threshold equals the chronic threshold, when accepting that generic 'adaptation to dose' (and not repair of injury) is non-adverse. The challenge to toxicologists and risk assessors is to unequivocally disentangle any possible adverse outcome from the adaptive response to high-dose liquid phase exposure conditions hardly to be found in human exposure scenarios. In summary, the objective of this paper is to identify any unifying metric and mechanisms which interrelate key physicochemical properties with the respiratory tract-specific toxicological outcomes from multiple inhalation studies on rats with the cVMS D4, D5 and D6.

2. Methods

Extensive regulatory, specialized mechanistic toxicity, and kinetic studies on D4 and D5 have been performed. This information, including the physicochemical properties, is readily accessible by download (DK-EPA, 2014; ECHA, 2020; SCCS, 2010; 2016). The toxicity studies addressing the acute, subchronic and chronic effects in rodents via dermal, oral and inhalation routes of exposure are reviewed in detail elsewhere (Jean et at., 2017a,b; Bridges et al., 2017; Dekant et al., 2017; Dekant and Klaunig, 2016; Klaunig et al., 2016; Burns-Naas et al., 1998; Franzen et al., 2017). The experimental studies on D6 are compiled in the ECHA (2020) registration dossier. The details of studies addressed in this analysis are made publicly available by SCCS (2010, 2016) (https://ec.europa.eu/health/scientific_committees/consumer_safety/docs/sccs_o_029.pdf). Methodological details such as group size, animal numbers/group, dose levels and modes of inhalation method applied, and key findings are detailed there. The studies referred to were executed by internationally recognized contract laboratories with an infrastructure suited to perform Organization for Economic Co-operation and Development (OECD) compliant inhalation studies (e.g., https://www.oecd.org/env/test-no-403-acute-inhalation-toxicity-9789264070608-en.htm; https://www.oecd-ilibrary.org/environment/test-no-412-subacute-inhalation-toxicity-28-day-study_9789264070783-en; https://www.oecd.org/env/test-no-413-subchronic-inhalation-toxicity-90-day-study-9789264070806-en.htm) following Good Laboratory Practices. Original reports were analysed in this comparative analysis (DCC, 1995a,b, 2004, Dow, 2005a,b; Dow Corning Corporation, RCC Group, 1995; RCC, 1995a,b).

Aspects regarding bioaccumulation received in-depth attention by pharmacokinetic analyses supplemented by physiologically based (PBPK) modelling (Andersen et al., 2008). The focus of this paper is on the inhalation route. These studies are from several laboratories using differing modes of exposure and equipment for the generation and characterization of test atmospheres. Conversions factors from 1 mg/m^3 to ppm are 0.0835, 0.0645, and 0.0541 for D4, D5, and D6, respectively.

2.1. Relative vs. absolute organ weights

Opposite to non-inhalation studies, food and water is withheld during exposure and handling for about or even more than ≈8 h. Additional overnight fasting periods prior to blood and specimen collection commonly are applied at terminal sacrifice with variable distance to the exposure-free weekend. Although not of widespread concerns, the impact of such methodological variables difficult to quantify may affect body weight gains in controls relative to treatment groups. Therefore, the analysis of absolute organ weight is given preference to any organ-to-body weight ratio depending on terminal body weights temporarily affected by the long fasting periods applied. The rat lung contains about 5 million migratory cells (mainly alveolar macrophages) that can be retrieved by bronchoalveolar lavage (Pauluhn, 2011). Any re-inflation of collapsed lungs by instillation of fixative into the airways may wash-away non-adherent cells. Hence, pathologists may score few remaining adherent AM out of five million macrophages. Despite this limitation, parenchymal pathologies can readily be identified, although subtle changes in septal thickness remain difficult to quantify due to variable lung fixation. Accordingly, absolute lung weight is given the highest diagnostic power for edema-related etiopathologies. It remains elusive as to how pathologists arrived at the isolated conclusion "alveolitis" in the absence of any consistent increased absolute lung weights. Recalling that the anticipated principal mode of action of liquid phase cVMSs is the functional disturbance in lung physiology controlled by Starling's and LaPlace's Laws, any increased lung water content (and lung weight) should be the most direct and quantifiable endpoint characterizing any physiological disturbances caused by adverse changes in the fluid dynamics of the lung (Li and Pauluhn, 2019).

2.2. Hypothesized unifying modes of action of the cyclic methylsiloxanes, D4, D5, and D6

Non-irritant lipophilic, surface active substances may change the physicochemical characteristics of mucin- and/or surfactant-containing/producing tissues of the respiratory tract. Therefore, regarding physicochemical interactions, the predestined locations for such interactions are the Goblet cells in nasal passages and the

pulmonary surfactant in the alveolar region. Each region has its unique features regarding local dosimetry and the ensuing localized response to inhaled dose. These features call for clear descriptors of any substance-specific "adversity" relative to generic "adaptive" (non-adverse) processes serving the well-being of the exposed species. Keeping in mind that the rat is evolutionary adapted to "dirty" environments, clean laboratory rats are fully protected from any normally occurring environmental challenges or sensory cues. Thus, inhalation exposure to any "cue" may trigger yet "dormant" functional response in a system evolutionary optimized to make it more tolerant to subsequent exposures in their normal habitat. No doubt, such adaptation to inhaled dose must clearly be distinguished from any maladaptation or even the adaptive repair of any preceding injury. This distinction is paramount for distinguishing generic "high-dose phenomena" from any substance-specific adverse response.

Accordingly, the magnitude of the exposure dose and related high concentration-specific phase examined in contrived inhalation studies must be related to that environment of humans observing species-, lesion- and regulatory-specific context. For instance, the number and pool-size of alveolar macrophages (AM) in clean control rats, body weight adjusted, is much lower than that of healthy humans (Pauluhn, 2011). The generic adaptive capacity of the AM-pool for engulfing and processing deposited aerosol and/or dysfunctional surfactant is remarkable. However, the mere adaptive increase in AM to inhaled dose often appears to be (mis)diagnosed as evidence of pulmonary inflammation which then triggers the regulatory label 'pulmonary irritant'. Consequently, an ostensive substance-specific outcome will then be GHS-classified as a **S**pecific **T**arget **O**rgan **T**oxicant (STOT) because of a generic adaptive response (https://unece.org/fileadmin/DAM/trans/danger/publi/ghs/ghs_rev06/English/03e_part3.pdf). GHS stands for the Globally Harmonized System of Classification and Labelling of Chemicals. GHS defines and classifies the hazards of chemical products and communicates health and safety information on labels and safety data sheets. All significant health effects that can impair function, both reversible and irreversible, following single exposure or repeated exposure, are included. Thus, lesion context and degree of adaptation are some of the most important but controversially discussed determinants in translational risk assessment. Lung overload phenomena and physiological exhaustion to dose often are categorized as "adverse" when not pursuing a holistic approach that takes account of the broader perspective (Palazzi et al., 2016). Therefore, any particular "lesion" should be considered as part of a constellation and not in isolation.

In studies with the cVMS addressed in this paper, the metabolically active olfactory epithelium at the level of the ethmoturbinates is exquisitely sensitive to irritation; however, no specific lesions were found at this location. The epithelium of the ventral apex of the larynx is known to be highly responsive to irritant aerosol as well. Again, evidence of irritation was absent. Thus, the absence of findings frequently observed as accompaniment following exposure to irritants, supports a *sensory* or *mechanical* causality rather than any cytotoxic irritant etiopathology of findings.

In the rat, six morphologically distinct cell-types are unevenly distributed along the mucosal surface of the nasal passages: goblet cells, ciliated cells, non-ciliated columnar cells, cuboidal cells, brush cells, and basal cells. Their relative location-specific abundance and morphology change species-dependently upon sensory stimuli. Goblet cells are most numerous on the nasal septum where they are intermixed with ciliated cells (Monteiro-Riviere and Popp, 1984; Rogers, 1994) . The increased abundance of these cells is commonly denoted as goblet cell hyperplasia., a response often seen in areas of the nasal cavity that are lined by transitional and respiratory epithelium. Goblet cell hyperplasia is thought to be an adaptive response of exposure to irritants or substances stimulating nociceptive receptors. The increased abundance of these cells appears to be a unifying finding noted in the response to inhalation exposure to lipophilic cVMS in their liquid phase. Aerosol deposition near the vomeronasal structures can elicit nociceptive sensory stimulations via the afferent trigeminal nerve-endings present in this region. The ensuing species-specific efferents serve protection through increased mucous production and secretion.

Liquid cVMS deposited at this location of the respiratory tract may not only elicit sensory stresses by physical mechanisms; mixed with mucins the rheological properties may change as well. Bearing in mind the small lumen of the airways of rats, any over-abundant protective mucous layers may affect functions not occurring in man because of much larger airways and differing anatomy. The opening of the nasolacrimal duct into the inferior nasal meatus of the nasal cavity is partially covered by a mucosal fold. Excess tears flow through nasolacrimal duct drains into the inferior nasal meatus. At face value, histopathological evidence suggests that liquid aerosol at high exposure levels impinge at nasal locations close to the vomeronasal system. For nonreactive volatile organic chemicals excellent correlations were found between log(RD_{50}) and log(vapor pressure) or Ostwald gas-liquid partition coefficient (Alarie et al., 1995). These findings support the hypothesis that the physicochemical properties of cVMS in their liquid phase could elicit nonspecific trigeminal sensations stimulating nociceptive responses. Along with these responses, ducts opening into the nasal cavity may become obstructed at overwhelmingly high concentrations liquid aerosol followed by secondary inflammation. The presence of associated lesions, such as inflammation or epithelial hyperplasia, is important as this helps to distinguish from non-specific responses and cytotoxic irritation. The latter can be ruled out because of the relative undisturbed matrix of cells adjacent to goblet cells found in cyclic siloxane-exposed rats. Goblet cells are normally also found in air-exposed control rats (Pauluhn and Mohr, 1999). Although speculative, it appears reasonable to assume that the anatomic differences of the nasal structures of rats and humans make the rat more susceptible to the chronic exposure of aerosol at concentrations exceeding those called for the limit test of acute inhalation studies.

Inhalation exposure to D4, D5, and D6 caused an increased incidence of a minimally increased AM-pool with/without foamy inclusions at the exposure level in the range and above Vsat. Apart from the high-level groups with intensity scores from minimal to moderate, the lower and intermediate exposure groups were most often scored borderline, that is minimal to slight. This renders comparisons difficult at 'borderline' intensity of findings in the absence of criteria differentiating 'histology' from 'histopathology' (Gibson-Corley et al., 2013).

The histopathological finding from the repeated exposure 13-week inhalation studies in rats with all three compounds were consistent with the aerosol phase-specific anticipated locations of predominant deposition. In rats exposed in the range and above Vsat to D6, the cyclic siloxane with the lowest Vsat and least tendency of spontaneous evaporation of aerosol, the very proximal nasal passages lined with squamous, transitional, and respiratory epithelium were hyperplastic as defined by the following findings: thickened epithelium with irregularly arranged cell layers, increased nuclear density and sometimes an increase in subepithelial glands areas of subacute inflammation. Hyperplasia of mucous (goblet) cells was characterized by increased mucin within the goblet cells, increased mucous cells within transitional epithelium, and frequently an increased number of mucinous subepithelial glands in respiratory epithelium. At locations with mucosa composed of both respiratory and olfactory epithelium, the only test article-related finding was mucous cell hyperplasia at the intermediate and high exposure levels. There were no test article-related microscopic observations in nasal sections of the more distal nasal passages or larynx (ECHA, 2020).

The inertia of large aerosol causes instant deposition in the more proximal nasal airways with vaporization within the more distal airways. At the alveolar level, the surface tension lowering surfactant conveys the forces to maintain physiological homeostasis following Starling's and LaPlace's Laws (Rhoades and Bell, 2013). Pulmonary surfactant consists of an amphiphilic layer predestined to retain substances with similar amphiphilic properties. Deposited liquid aerosol

sharing this property is suited for causing changes in the dynamic interfacial properties of this viscoelastic boundary layer. Surfactant homeostasis is maintained by discharge of surfactant from type II pneumocytes while dysfunctional surfactant is recycled by alveolar macrophages. Depending on the air:blood gradient of partial pressures, the vapor phase cVMS are absorbed or exhaled from the lung. At the first glance, the vapor phase-specific equilibrium of exhaled cVMS does not affect the function of pulmonary surfactant. In contrast, inhaled cVMS in the liquid phase are relatively immiscible liquids in aqueous systems but may readily interact with or emulsify amphiphilic alveolar surfactant where one liquid is dispersed throughout another liquid.

At Vsat the gaseous phase coexists with the liquid phase dynamic interfacial tensions in meso-equilibrium at a given temperature and dynamically changing size of the alveolus. The resulting emulsions may become thermodynamically unstable and may separate into two phases over a period of time diminishing the physical performance of the alveolar surfactant layer. Typically, such changes follow a sigmoidal interrelationship where increased dysfunctional alveolar surfactant can be expected with increased deposited liquid aerosol (Rosen, 2004). When recognizing the intrinsic task of AM to endocytose and degrade such micellar structures, it comes as no surprise that cVMS mixed with surfactant are dealt with like dysfunctional pulmonary surfactant. Therefore, any adaptive process showing phenotypes of enlarged and/or increased numbers of AM with/without foamy inclusions (phospholipoproteins) reflect an entirely benign and adaptive process to maintain alveolar stability and function as long as maladaptation can be prevented. Lysosomal trapping and ensuing abnormalities are not expected to occur for this class of biodegradable substances.

Collectively, none of the inhalation studies with D4, D5 or D6 demonstrate conclusive histopathological evidence suggestive of respiratory tract irritation. The anatomical arrangement of the upper airways of rats allows the larynx to be exposed to higher concentrations in rats compared to humans (for more details see DeSesso, 1993; Weber et al., 2009). The laryngeal epithelium did not show any sequelae suggestive of irritation. Accordingly, reported histopathological changes in the proximal nasal passages appear to be caused by non-specific adaptation to an overwhelmingly high deposited dose of liquid aerosol rather than any site-of-deposition specific susceptibility or adversity. The adaptations observed are entirely plausible for the protective responses occurring in the proximal nasal passages of rats. It is important to recall that this location is rich in nociceptive innervation and sensitive to rodent-specific nociceptive afferent neurogenic stimuli. If occurring, they are stereotypically characterized by findings suggestive of sensory trigeminal stimulations and ensuing stress-related accompaniments such as stimulation of the hypothalamic-pituitary-adrenal-thymic axis (Pauluhn, 2018a; Smith and Vale, 2006). All-in-all, in rats, such stimuli may cause rodent-specific nociceptive stress-related changes typically manifested by increased adrenal weights and decreased thymus weights (Pauluhn, 2018a).

2.3. Variables affecting inhalation dosimetry

The complex interplay of nucleation and aerosol condensation together with aerosol growth may produce a “coagulation sink” leading to “condensation aerosol” (Hinds, 2011; Ho, 1997; Sosnowski and Odziomek, 2018). Once coagulated, the temporal stability of aerosol increases with increased aerosol growth even below V_{sat}. Especially in whole-body chambers, the spatial lack of homogeneity of aerosol is a likely occurrence. “Hot spots” of aerosols may cause variable intensities of portal-of-entry related effects. Unlike whole-body exposure, the short residence time prevailing in nose-only chambers might be insufficient to allow complete re-equilibration of phases upon dilution. Atmospheres believed to be free of aerosol below V_{sat} may still contain liquid aerosol commonly deposited at higher surface doses than vapors which are retained by diffusion and partition. Local dosimetry is affected by the specific physicochemical property of the interface lining the airways.

The deposition of a liquid aerosol of a semi-volatile substance will be highest at the coolest part of the airways which are those of the nostrils. However, when inhaled, the gradient of V_{sat} changes precipitously as can be deduced from the thermal mapping of the airways (data related to the end-inspiratory temperatures measured in humans breathing room air; for details see McFadden et al., 1985). The most proximal measured temperature was 32 °C, with temperatures at the tracheal carina and the most distal thermistor of 33.2 and 35.5 °C, respectively. As ventilation increased, the temperature progressively decreased at each location. The transposition of this data from humans with normally 15 breaths/min and a VE (ventilation) of 20 L/min to rats with 130 breaths/min and a VE of 0.2 L/min and lung weights differing by 2-3 orders of magnitude preclude any precise extrapolation. Nonetheless, these findings demonstrate that during the conditioning of inspired air in the extrathoracic and intrapulmonary airways profound thermal changes extend into the periphery of the lung (McFadden et al., 1985). The rats’ kg-adjusted ventilation is ≈ 4-times higher than that of humans (Bide et al., 2000). Due to the much shorter distance to travel at higher flow rates and smaller airway lumens, 32 °C is taken as a reasonable (over-) estimate of the temperature present in the intrapulmonary airways of rats. One additional important species difference between human and rats is the nociception-related difference in thermoregulation (Gordon, 2005; Gordon et al., 2007; Pauluhn, 2018a). While humans are homeothermic, rodents are thermolabile. As discussed in greater detail elsewhere (Pauluhn, 2018a), rats may undergo nociceptive hypothermia as low as ≈32 °C following inhalation exposure to sensory stimulants. Hence, regarding the high-dose phenomena caused by the liquid aerosol phase, thermolabile rodents are inherently more susceptible than humans. From that it would seem reasonable to assume that the respiratory tract of humans facilitates evaporation of aerosol whereas the more monopodial lung of rats minimizes aerosol vaporization. This supports the notion that the high-dose portal-of-entry phenomena observed in rats are unlikely to occur in humans.

2.4. Acute inhalation toxicity and metrics of dose-effect relationships

Pulmonary surfactant serves mechanical functions of the lung, i.e., preventing expiratory atelectasis and permeability edema because of imbalanced hydrostatic pressures from the vascular site counterbalanced by the protein-oncotic pressure from the alveolar site (Starling’s Law). Liquid-liquid siloxane-surfactant interactions deploy the known surface-spreading characteristics of these substances with changes in surface tension of surfactant. According to LaPlace’s Law (Rhoades and Bell, 2013, 2013, 2013) with $P = 2\ T/r$ (P stands for the transpulmonary pressure keeping the lung and alveoli inflated, r is the radius of an alveolus, and T representing the tension at the alveolar interface). Depending on the total retained mass of liquid aerosol of a surface-active substance relative to the pool of surfactant present in the lung, alveolar instability is a likely sequel. If markedly affected, atelectasis accompanied by increased transendothelial fluid transudation into the alveolus is a likely forecast to occur in acute inhalation studies aiming at lethal outcomes. The rate at which fluid is filtered across vascular endothelium (transendothelial filtration) is conceptualized by Starling’s law (Rhoades and Bell, 2013). As explained earlier, this equation describes the involved forces, i.e., drop in the protein-oncotic counter-pressure to the intracapillary hydrostatic pressure, in mathematical terms. Collectively, irritation-like phenomena are not required for outcomes entirely controlled by physical equilibria.

Pneumocytes type II synthesize and store pulmonary surfactant. The turnover time of its components is short and variable depending on the degree of physiological adaptation (Wirkes et al., 2010). Recalling that surfactant is a complex mixture with disaturated-phosphatidylcholine (DSPC) as the main component, DSPC often is often chosen as landmark of surfactant kinetics. As the DSPC pool size may change with increased synthesis, rat-specific figures could not be found; however, appear to be in the range of about 1–10 h. Thus, at non-exhaustive

exposure levels, acutely depleted surfactant is expected to be homeostatically replenished without any carry-over from one exposure day to the next. This supports the mechanistic hypothesis that adaptive outcomes should follow a metric dependent on the $C_{aerosol}$ x t per exposure day, independent of the frequency of day-by-day exposures. Hence, the 'acute-on-chronic' aggravation of injury typically occurring following repeated exposure to airway irritants, is incompatible with this mode of action. Bearing this hypothesis in mind, one should expect a clear-cut increase in the acute pulmonary toxicity of cVMS when using concentrations exceeding V_{sat}. This suggests a proportionality of the non-lethal threshold concentration (LC_{01}) and median lethal concentration (LC_{50}) with V_{sat}. This relationship holds as long the C x t dose at any specific V_{sat} is high enough to cause over-proportional physicochemical disturbances within the pool of surfactant. This analysis supports the notion that longer chain cVMS $\geq$ D7 with even lower V_{sat} are acutely less potent than predicted since the buffering pool of surfactant becomes proportionally larger relative to the inhaled C x t of deposited siloxane.

Unlike aerosols, for poorly water-soluble, non-reactive vapors, dosimetry becomes perfusion-dependent for systemic targets after attaining steady-state. Due to its low water-solubility, the vapor rapidly saturates the aqueous layer lining the airways. Retention of the vapor phase in airways appears to be minimal to negligible in both obligate nasal breathers (e.g., rats) and oronasally breathing humans. This means, with extended exposure duration and attainment of steady state, the major fraction of the inhaled vapor (≈95%) will be exhaled unchanged again as hypothesized and reported by Franzen et al. (2017). Some fraction of the aerosol deposited in the alveolar region may gain access to the systemic circulation following a ventilation-related absorption.

Although some of the physicochemical characteristics of these cVMS may argue for bioaccumulation, this is unlikely to occur for a vapor exhaled unmetabolized and at a very fast rate (Andersen et al., 2008). This renders lipophilic sinks to become intermediary storage compartments with limited, if any, likelihood for bioaccumulation. To verify/refute this misperception, the analyses given in the Results and Discussion section compare outcomes normalized per-day support non-cumulative outcomes. As typical for inhaled substances locally acting in the pulmonary region, adverse outcomes are determined by a per exposure day C x t-related metric. If "adversity" is caused by the acute C x t-related depletion of surfactant, the rapid turnover of surfactant prevents any carry-over of adversities following recurrent exposure as suggested above.

2.5. Non-specific sensory nociceptive stimuli

Stimulation of the rodent-specific nociceptive sensory system may cause neurogenic-triggered stresses with somatizations which often are misjudged as evidence of systemic toxicity. It can be expected that the high lipophilicity of the liquid aerosol phase of cVMS undergoes partitioning with the lipophilic membranes of directly exposed nerve endings. The most stereotypical response in rodents to such stimuli is hypothermia, bradypnea, bradycardia and stress-related changes of stress-organs (see Pauluhn, 2018a). In rats, a hibernation-like state can be produced by attenuating thermogenesis (Jinka et al., 2015). Thus, rats may be able to adapt to environmental changes in their natural habitat by reducing their energetic needs by torpor, a reversible state of suppressed metabolic demand, decreased ventilation, and decreased body temperature. This is achieved by an autonomic or forced hypothermia and behaviorally regulated hypothermia aimed at diminishing the rats' physiologic requirements as a short-term strategy to match stressful conditions (Gordon, 2005; Jinka et al., 2015).

2.6. Data analysis

SigmaPlot 14.0 software (Systat Software Inc., Point Richmond, CA) was used for graphical analyses. The median lethal concentration (LC_{50}) was re-calculated using the Probit method-based algorithms (Fortran) as published by Rosiello et al. (1977). The non-lethal threshold concentration (LC_{01}) was calculated from the regression parameters at 1% mortality. In lieu of the NOAEL, preference was given to the BMCL, which is the one-sided 95% lower confidence limit of the benchmark concentration (BMC) of the most salient dose-limiting adverse endpoint. The calculated BMCLs values are taken as Points of Departure (PODs). Continuous data sets were analyzed by the Hill model which fit the data best in relation to the suite of other models provided by the U.S.-EPA benchmark software (Version, 2.7.0.4., default selection for continuous data (BMR, S.D.); for a more detailed description of this software see U.S.-EPA (2002, 2015)).

3. Results and Discussion

3.1. Methodological pitfalls of testing semi-volatile compounds

Depending on the specific physicochemical properties of the liquid phase, alveolar dysfunction occurs by entirely non-irritant physical interactions as delineated above. The studies referred to in this analysis used nebulizers or evaporators for test atmosphere generation. Nebulizers produce mixtures of vapor and dispersion aerosols which tend to coagulate with particle growth at concentrations exceeding V_{sat} or to evaporate at concentrations lower than V_{sat}. The vapor-aerosol equilibrium changes appreciably from the high concentration atmospheres present at high temperature in the generator and after dilution with carrier air at room temperature prior to entering inhalation chambers. When using piston pumps transferring a liquid test substance from a reservoir into any heated evaporator filled with glass beads to increase surface area and shear forces, a vaporizer could readily become an atomizer. Feeding tubes ducting liquid test substances with high vapor pressures into a heated vaporizer may generate intermittent expulsions as approaching the irradiation heat of the vaporizer. Depending on the stroke volume and pump frequency, an intermittent vaporization profile with high peaks of liquid aerosols may occur within the generator; however, this intermittent exposure profile remains undetected by time-weighted averaged sampling of test atmospheres (Pauluhn and Mohr, 2000). As this happens prior to dilution, such conditions favor nucleation and particle growth. For instance, the Vsat of D5 at 22 °C equals ≈2000 mg/m^3. At the temperatures present in the vaporizing systems of 30, 50, and 80 °C, the saturation vapor pressures yield ≈4,000, ≈18,500, and ≈113,000 mg D5/m^3 at the low, intermediate, and high concentration, respectively. In other words, at 22 °C in the inhalation chamber, dilutions must be instant and more than 50-fold in the high group to prevent condensation aerosol from occurring.

When applying the same rationale to the less volatile D6 with a vaporizing system maintained in the range of 100 °C (Vsat ≈100,000 mg/m^3) and inhalation chamber Vsat at 22 °C (Vsat ≈278 mg/m^3), the ≈350-fold supersaturation may result in appreciable amounts of condensation aerosol to be present prior to entering the inhalation chamber. Keeping in mind that the lifetime of aerosol is dependent on the equilibrium vapor pressure and the tendency of a liquid phase to escape into the gas phase by vaporization, such a temperature gradient decreases not only the vapor pressure but also evaporation rate (Mackay and van Wesenbeeck, 2014). Thus, despite the dilution of atmospheres at the chamber inlet, the lower vapor pressure of D6 may increase the residence time of condensation aerosol in a dynamic equilibrium (at quasi state). From that it cannot be ruled out that exposure atmospheres well below the Vsat of D6 may still contain aerosol with spatial gradients within inhalation chambers. To reiterate, such quasi-steady state conditions experienced in dynamic inhalation chambers cannot be reproduced under the practices of real-life exposures to these substances in open systems.

This conceptional analysis supports the conclusion that the highly supersaturated conditions present in the ducts to inhalation chambers prior to dilution may substantially increase the lifetime of larger

droplets. These may be stable enough to cause inhomogeneities of vapor-to-aerosol ratios from chamber inlet and downstream in the inhalation chamber. These methodological pitfalls are indispensable high-dose level events consistent with the conclusions of renowned expert panels (SOT, 1992). It was concluded that the physical laws for the aerosol particle size to be used in acute inhalation studies limit exposure atmosphere at a concentration in the range of 5000 mg/m^3. Hence, the dynamics of aerosols at such concentrations and above, this limit can be deemed to be "uncontrollable" both in terms of physical and physiological laws. In the context of the siloxanes addressed in this paper, the physical property "aerosol" translates to liquid-specific characteristics, e.g., stickiness, surface tension or amphiphilicity and mixability with other liquids to mention but a few. The vapor phase is devoid of these characteristics. This calls for physical phase-specific risk characterization and assessment.

3.2. Impact of vapor saturation on endpoints of toxicity

Ranges of extrapolated vapor pressures (P) of D4, D5, and D6 were published by Brooke et al. (2009a-c). These authors fitted vapor pressure data, measured using an ebulliometer, from Flaningam (1986) to both the Antoine and the AIChE DIPPR equations (for abbreviations and methods see Brooke et al., 2009a-c). These semi-empirical equations are mathematical constructs derived from the Clausius-Clapeyron relation between the vapor pressure and temperature. The given data base addresses temperatures from 88 (≈175), 110 (≈210), and 139 °C (≈245 °C) up to the boiling points (values in brackets) of D4, D5, and D6, respectively. Although the relation between vapor pressure and temperature is non-linear, the applied methods use a logarithmic scale to produce slightly curved lines. Due to the higher parameterization of the Antoine and especially the DIPPR equations, relative to the August equation (*vide infra*), better curve fitting can be expected by the DIPPR equation. However, this does not necessarily resolve the uncertainties associated with extrapolations of vapor pressures from the medium-pressure range to the lower atmospheric pressures prevailing at ambient temperatures. Although beyond the scope of this review, it could be argued that any relationship of vapor pressures and the calorimetric enthalpies of vaporization could more exactly be estimated if observing the thermodynamic constraints linking vapor pressures and the thermal data between heat capacity of an ideal gas and that of the liquid available at lower temperatures (Růržička and Majer, 1996).

The P's used in this paper for calculating Vsat are extrapolated to match P's from 15 °C to 35 °C. Brooke et al. (2009a-c) reported pairs of P-values for each method at 20 °C and 25 °C. These served as basis for calculating the exact slope and intercept of the line defined by (log P_1, $1/T_1$) and (log P_2, $1/T_2$) as defined by August's method which describes the linear relation between the logarithm of P and the reciprocal absolute temperature (T) (in Kelvin). Subsequently, these slope and intercept parameters were used to re-calculate the method-specific P's within the range of interest (Fig. 1). The different method-specific outcomes call for cross-validation using a P at 25 °C as published by Lei et al. (2010). These authors applied the isothermal retention times of seven cVMS using alkanes as standard reference and calibration. As demonstrated in Fig. 1, the Antoine's equation-derived vapor pressures reproduced best those from Lei et al. (2010). Accordingly, the Vsat's calculated by this method are most reliable for use in risk characterization. Within the given range of temperatures, the vapor saturation concentrations re-calculated by the August's method are sufficiently coherent with those using the original parameterization of Antoine's equation as published by Brooke et al. (2009a-c) (Fig. 1, dashed lines) if 35 °C is not exceeded. Accordingly, for the Vsat's exceeding this temperature, the Antoine's instead of the August's equation was used.

Based on the relationships given in Fig. 1, the concentrations at Vsat estimated to prevail in the intrapulmonary airways of rats (≈32 °C) yielded 25, 4.6, and ≈0.9 g/m^3 for D4, D5, and D6, respectively. The 4-h LC_{01} values of D4 and D5 shown in Fig. 2 yielded 23 and 4.6 g/m^3, respectively. This outcome is in remarkable accordance with the mechanistic hypothesis of this analysis, namely that only the liquid-phase of these cVMS can convey the physicochemical properties causing instability of pulmonary surfactant. In fact, when comparing these two phases, the molecules in a vapor phase are diffusely distributed over the large surface area of the lung with small mass/cm^2, whereas the molecules in a liquid phase produce a much higher focal mass/cm^2 and focal disturbances. As already referred to in the preceding sections, this mode of action becomes operative proportional to the mass of deposited aerosol and proportional homeostatic countermeasures of the pulmonary system. The following series of events can be anticipated: (1) depending on the relationship of the deposited liquid phase, cVMS

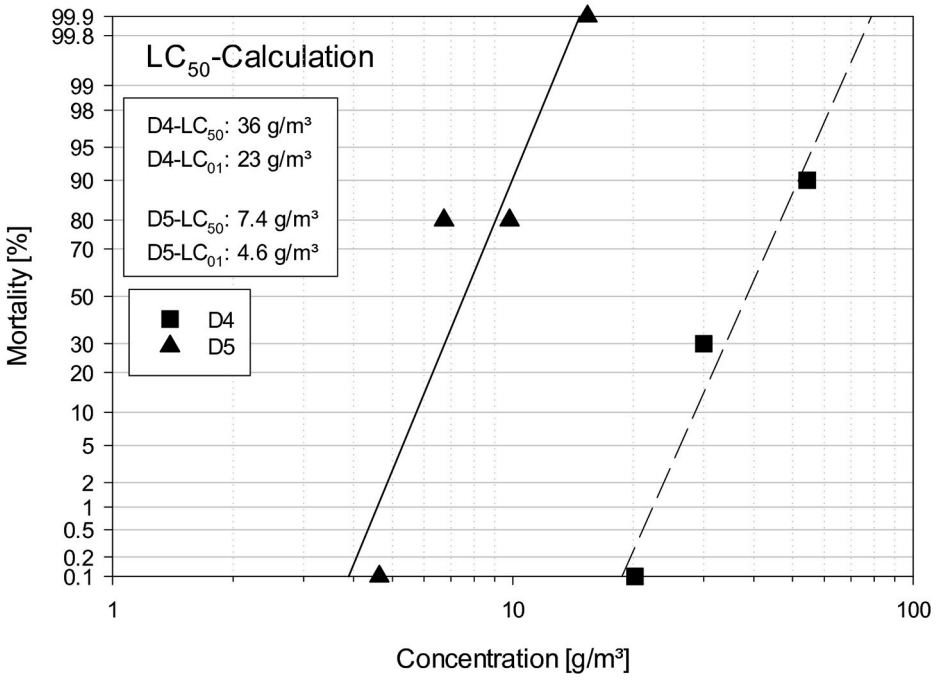

Fig. 2. Probit scaled mortality data from acute 4-h LC_{50} studies on 5 rats/group/sex were recalculated (male and female rats combined) by Probit analysis according to Rosiello et al. (1977). Infinite Probits at 0% and 100% mortality are presented and calculated as values of 0.1% and 99.9% mortality. The median lethal concentrations (LC_{50}) and non-lethal threshold concentrations at 1% mortality (LC_{01}) were calculated by regression analysis. Mortality data were reproduced from SCCS (2010, 2016). Experimental details of these OECD-TG#403 compliant studies (https://www.oecd.org/env/test-no-403-acute-inhalation-toxicity-9789264070608-en.htm) are given in the SCCS appraisal.

relative to the pool of pulmonary surfactant, dysfunctional surfactant is produced with re-equilibration by type II pneumocytes as inhaled; if decompensated, a permeability lung edema occurs; (2) the precipitated complexes of pulmonary surfactant and cVMS undergo endocytosis by AM engaged in the catabolism of worn-out surfactant. This process is entirely benign and physiological. (3) with increased load of liquid phase cVMS, the homeostatic response is to increase the pool size of AM as required to maintain the physiological functions of the lung; (4) the relationship of AM and polymorphonuclear neutrophils (PMN) parallel each other (Pauluhn, 2018b); as long as 'danger signals' are not triggered by AM, PMNs act as phagocytes but not as inflammatory cells. They come and go in parallel to the population of AM. (5) This sequence of events requires a balanced interpretation of findings: (i) lung weights need to be dose-dependently increased; (ii) at steady state, adaptive effects reach steady state as well, and aggravation following re-exposure does not occur.

The thermodynamically calculated threshold concentrations of cVMS characterizing the attainment of Vsat in the lung shows coherence with the time-adjusted non-lethal threshold LCt_{01} (Figs. 1 and 2). The precipitous increase of mortality in the acute LD_{50} studies of D4 and D5

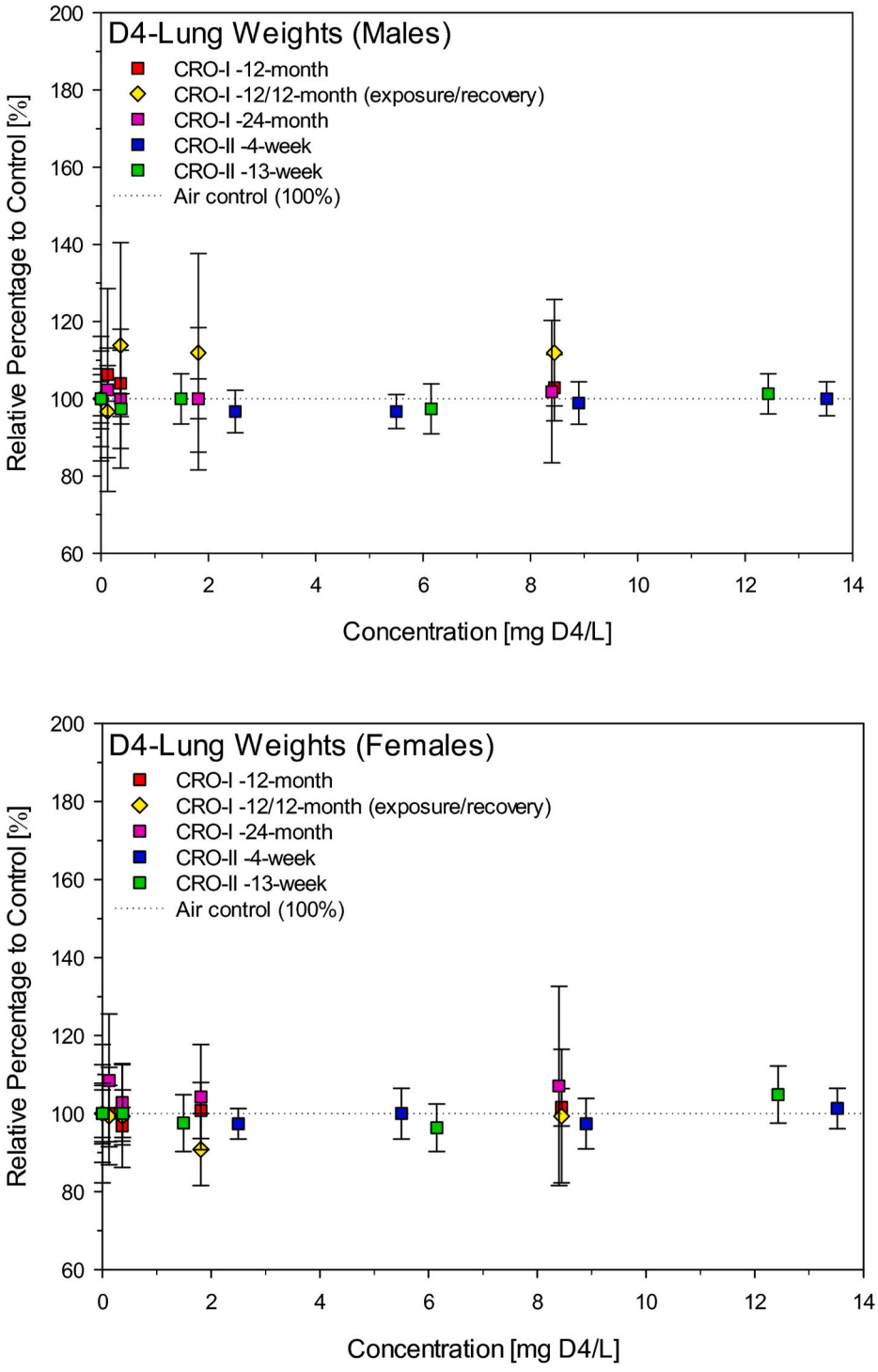

Fig. 3. Comparative analysis of absolute lung weights from rats exposed to D4 for 1- to 24-months. These studies were from two different contract laboratories (CROs). Data points represent means ± S.D. of 10–20 animals/group/sex (for details see SCCS, 2010; 2016).

in rats following exposure to increasing concentrations above Vsat can be taken as robust evidence that only the liquid phase equilibrates and interacts with pulmonary surfactant. Any major demulsification of pulmonary surfactant deteriorates the tightly tuned physical factors stabilizing the pulmonary blood-air barrier. If severe enough, an acute permeability edema occurs. This metric follows a concentration$_{aerosol}$ x time ($C_{aerosol}$ x t) paradigm reflecting the respirable dose of aerosol deposited in the surfactant layer of alveolar region. Any quantitative assessment of aerosol at the pulmonary level remains complex and speculative because multiple highly dynamic equilibria must be accounted for. Functional surfactant appears to be titrated out proportional to $C_{aerosol}$ x t following exposure to the cyclic siloxane. The hypothesis articulated that carry-over of adverse effects from one exposure day to the next are unlikely to occur was shown to be fulfilled in Figs. 3–6.

Acute–on-chronic effects are frequently observed outcomes in inhalation studies with airway irritants (see Pauluhn et al., 1995). To test this hypothesis, liver and lung weights were compared as integrating endpoints of systemic and local effects. To adjust inhalation studies from 1-month to 2-years exposure durations to a unifying acute metric, these markers of effects were normalized to those of the time-matched concurrent control of each study (= 100%) vs. the averaged nominal concentration of study (Figs. 3–6). For D4, sex-specific differences in lung weights were not apparent (Fig. 3). Therefore, in all subsequent comparisons findings from both sexes were illustrated without sex-specific differentiation. When acknowledging the 100 ± 20% single standard deviation observed in controls, consistent concentration- or study duration-dependent changes in absolute lung weights were not apparent for D4, D5, and D6 (Figs. 4–6). In D4-exposed rats absolute liver weights increased concentration-dependently but not study duration-dependently. Unlike D4, concentration- or study duration-dependent changes in absolute liver weights did not occur following exposure to D5 and D6 (Figs. 4–6). These relationships appear to suggest that all findings are non-cumulative and dependent on the concentration per exposure day (dose rate). In seems that volatility – along with the higher concentration – determines systemic

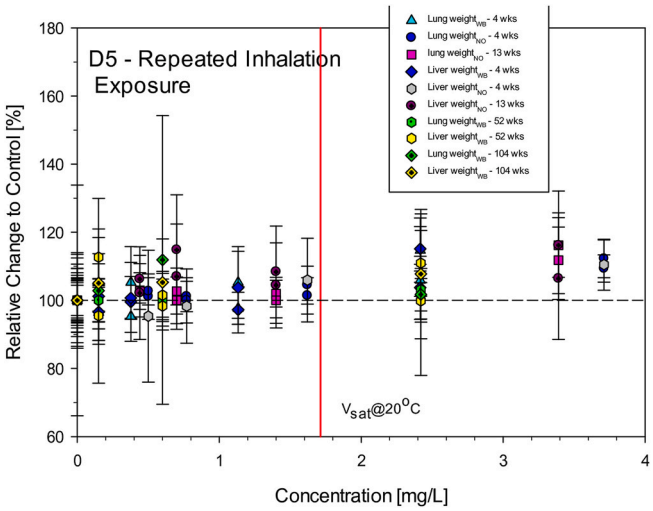

Fig. 5. Comparative analysis of absolute lung and liver weights from rats exposed to D5 for 1- to 24-months. Subscripts: NO for nose-only mode of exposure, WB for whole-body exposure. Data points represent means ± S.D. of 10–20 animals/group/sex (for details see SCCS, 2010; 2016). The vertical red line indicates the concentration of V_{sat} at 20 °C. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

bioavailability and increased uptake and turn-over of lipids and/or lipophilic cVMS in the liver. Kinetic analyses revealed "deep compartments" to describe the time-course kinetic behavior of these cVMSs in the liver (McMullin et al., 2016). The authors consider this deep tissue compartment a storage depot from which the free parent chemical is released first to the tissue itself and then to systemic circulation. Similarly, the 13-week inhalation study with D4 on rats revealed increased liver weights as well; however, without histopathological evidence of hepatotoxicity (DCC, 1995a). Hence, increased hepatic weight appear to

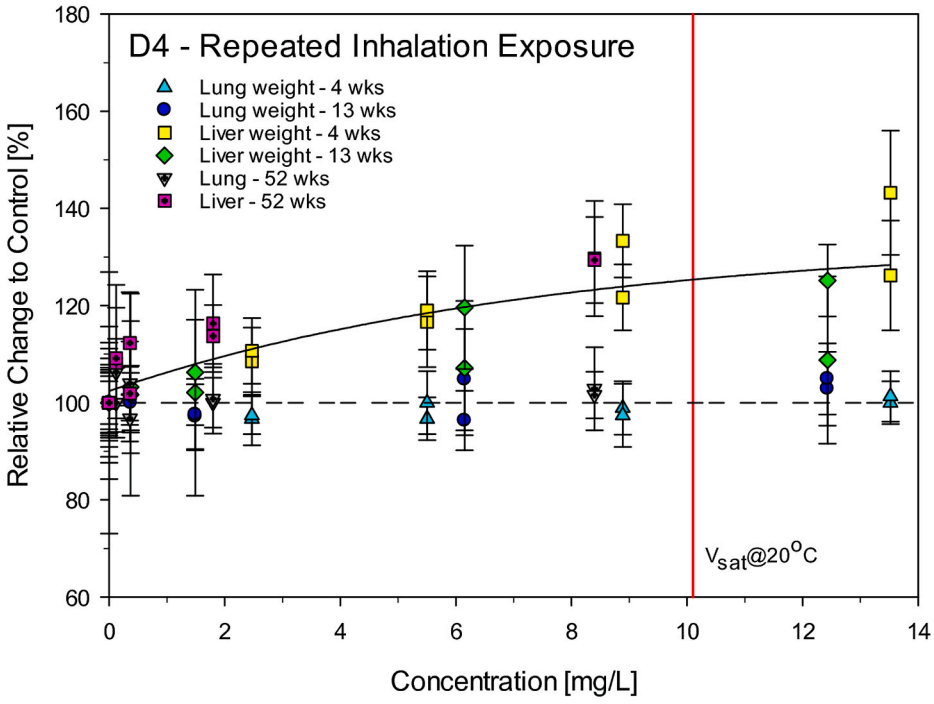

Fig. 4. Comparative analysis of absolute lung and liver weights from rats exposed to D4 for 1- to 12-months. Nose-only mode of exposure: up to 13-weeks, whole-body exposure: 52-weeks. Data points represent means ± S.D. of 10–20 animals/group/sex (for details see SCCS, 2010; 2016). The vertical red line indicates the concentration of V_{sat} at 20 °C. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

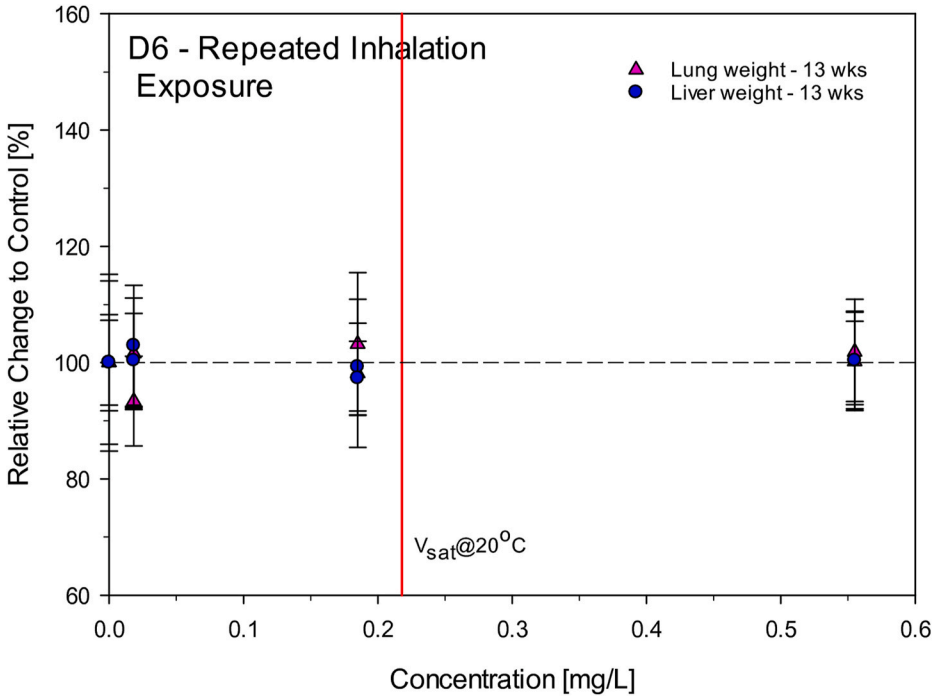

Fig. 6. Comparative analysis of absolute lung and liver weights from rats whole-body exposed to D6 for 3-months. Data points represent means ± S.D. (for details see SCCS, 2010; 2016). The vertical red line indicates the concentration of V_{sat} at 20 °C. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

be dispositional in nature rather than adverse.

The predicted trend of increasing potency towards lung edema at concentrations above Vsat could be demonstrated for the acute lethality studies (Fig. 2) and repeated exposure inhalation studies experiencing inadvertent excursions at the very early phase of study above Vsat (see next section). None of these findings occurred at concentrations below or in the range of Vsat.

When following this concept, rats exposed to D4, D5, and D6 unequivocally meet the criteria for an entirely homeostatic response as illustrated in Figs. 3 and 4. This coherence is taken as evidence that pulmonary toxicity increases precipitously at concentrations containing the cVMS in the aerosol phase. Thus, to attain mortality, concentrations must be high enough to stabilize the thermodynamic equilibrium of aerosol present at ≈32 °C. Likewise, assuming inhalation chamber temperatures at ≥20 °C, aerosol-specific adaptive responses in the proximal nasal airways cannot be excluded at concentrations at ≥10,000 mg D4/m^3, ≥1700 mg D5/m^3, and ≥224 mg D6/m^3. In summary, the outcome of this analysis supports the conclusion that the nasal adaptive response or any potentially destructive pulmonary adversities require a substantially supersaturated liquid phase of cVMS to be present. It is highly doubted that such an equilibrium can be attained under conditions prevailing even at worst-case exposures of humans.

3.3. Acute-on-chronic outcomes - lethality

In the acute inhalation studies with D4 and D5 rats were exposed for 4 h to concentrations of 20120, 30030, and 54470 mg D4/m^3 with mortality 0, 30, and 90%, respectively. At lower concentrations mortality occurred within 3 post-exposure days whereas at the high exposure level animals succumbed during or shortly after exposure. Signs suggestive of acute lung injury included tachypnea, rales, and red nasal discharge. Gross necropsy of the animals with unscheduled deaths showed reddish foci in some tissues and increased lung and spleen weight, including involution of thymus. Discolored lungs were recorded in rats sacrificed at the end of the 2-week post-exposure period. Exposures to D5 were to 4640, 6730, 9820, and 15370 mg D5/m^3. No animals died at the lowest concentration, whereas 80% and 100% of rats died at the intermediate and top exposure level. In animals with unscheduled mortality, gross necropsy revealed red lungs partly collapsed while no macroscopic observations were recorded at the end of the 2-week post-exposure period (SCCS, 2016). Taken together, the onset of mortality occurring at high exposure levels is a typical characteristic of substances titrating out pulmonary surfactant with death as dictated by Starling's and Laplace's laws (Rhoades and Bell, 2013). This is complemented by observational descriptions suggestive of atelectasis followed by edema. Rats succumbing during the first exposure days, is a typical occurrence of pulmonary edema. Thus, for both cVMSs, mortality is caused by a physicochemical mode of action (direct interaction with pulmonary surfactant) rather than by any irritant etiopathology which would have called for signs of upper respiratory tract irritation and more delayed patterns of mortality (Pauluhn, 2019).

Recalling the null-hypothesis of this analysis, that assumes that the inhaled C × t of cVMS per exposure day determines the proportional loss of pulmonary surfactant per exposure day. This loss in surfactant is compensated by re-synthesis up to the next exposure day. Thus, all effects appear to depend on the dose delivered per exposure day rather than any cumulative exposure. However, it is prudent to recall that fluctuations in concentrations, phase, and temperature gradient in inhalation chambers cannot entirely be excluded so that effects may have been modulated by yet unknown peak concentrations and not necessarily by the measured time-weighted average concentration. Repeated inhalation exposure uses a 6-h instead of the 4-h exposure period. Accordingly, the 4-h LC_{01} of 23000 mg D4/m^3 and 4600 mg D5/m^3 must be adjusted to the 6-h equivalent of 15300 mg D4/m^3 and 3100 mg D5/m^3. The 4-week, 5 x 6-h/day study on rats with D4 began with the maximum nominal concentration of 16340 mg/m^3 (3 exposures) followed by a reduction to nominal 13100–14600 mg/m^3 the week beginning with day 6. Rats were found dead (4 of 20) on days 2, 6, and 7 which comes as no surprise because the 6-h adjusted LC_{01} was already exceeded. The continued exposure for 3-weeks at 13520 ± 570 mg/m^3

was tolerated without mortality. The 4- and 13-week repeated inhalation exposure studies with D5 used maximum concentrations of 2400 mg/m^3 (whole-body, 7-days/week for 4-weeks), 3700 mg/m^3 (nose-only, 5-days/week for 4-weeks), and 3400 mg/m^3 (nose-only, 5-days/week for 13-weeks) without any mortality. When considering differences between both nominal and actual concentrations and inhalation chamber temperatures the overall outcome is consistent. Thus, regarding the key factors for lethal effects to occur, a clear association of the Vsat of D4, D5, and D6 and potentially adverse outcomes could be established.

3.4. Acute-on-chronic outcomes – local and systemic targets

Based on the hypothesis articulated above, the chronic lung-related outcome from repeated inhalation exposure studies is expected to be contingent upon the acute functional disturbances of surfactant. If adaptive, this occurs in the absence of any cumulative toxicity. If, adverse, recurrent exposure of any predisposed location within the respiratory tract should manifest aggravated outcomes. AM are involved in surfactant homeostasis and uptake of materials foreign to the lung. Macrophages may contain foreign material consistent with the test

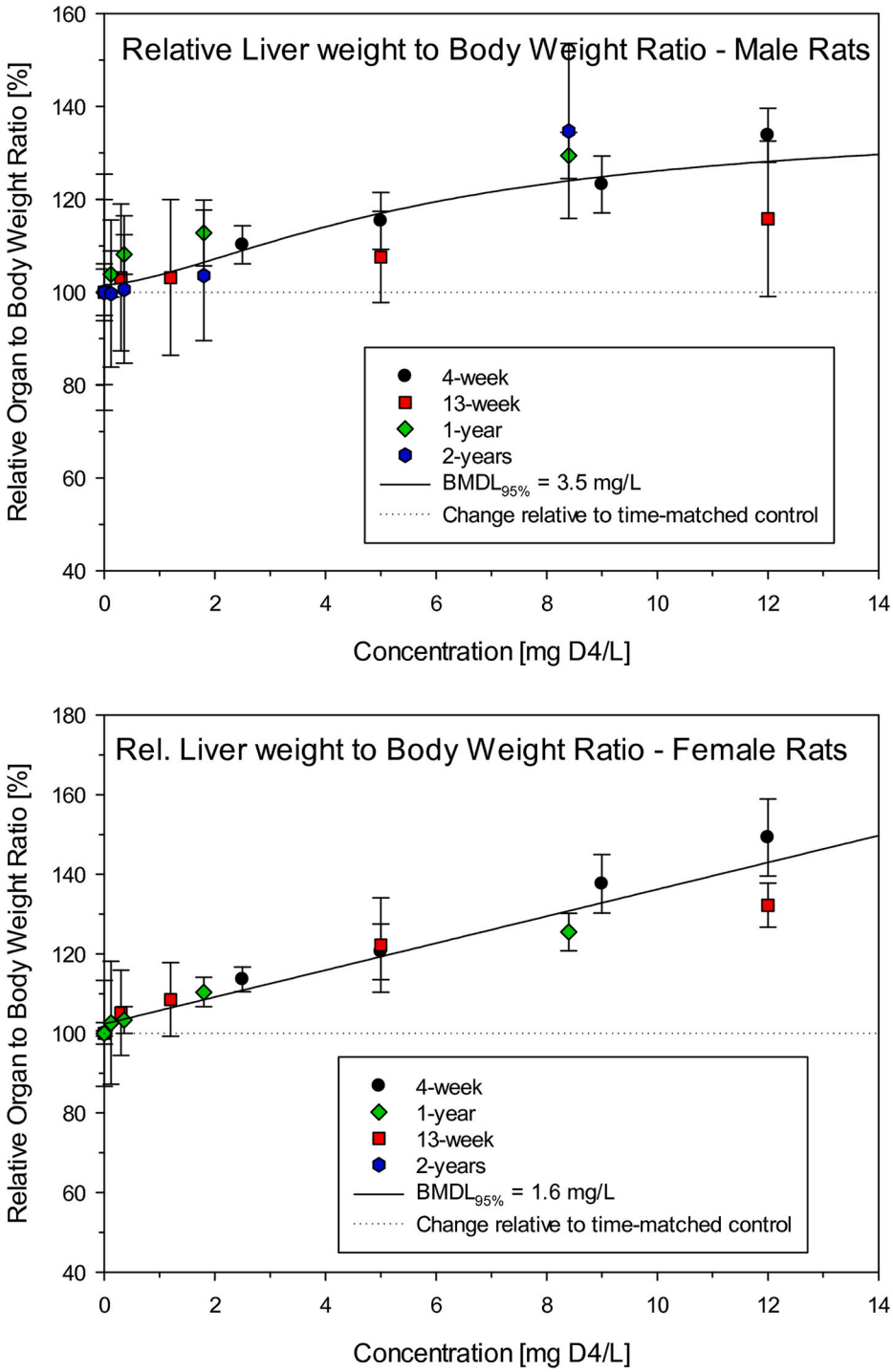

Fig. 7. Benchmark analysis of liver weight to body weight ratios from D4-exposed male (top) and female (bottom) rats covering exposure periods from 1- to 24-months. Data points represent means ± S.D. of 10–20 animals/group/sex (for details see SCCS, 2010, 2016).

article or its complexes with precipitated surfactant. What appears to "accumulate" is the adaptive increase of the pool of alveolar macrophages engulfing dysfunctional surfactant. Their normal elimination half-time of non-biodegradable material from the lung of rats is about 70 days (Pauluhn, 2011, 2014a,b). However, dysfunctional surfactant, possibly complexed with siloxanes, is expected to undergo biodegradation much faster.

For edema-inducing substances, the amount of water contained in the lungs has been shown to be the key marker of adversity in both acute and repeated exposure inhalation studies on rats with irritant vapors and aerosols (Pauluhn, 2000a,b). In humans, the EVLWI (extravascular lung water index) corresponds to the sum of interstitial, intracellular, alveolar and lymphatic fluid. An increase in the EVLWI is the pathophysiological hallmark of hydrostatic pulmonary edema and acute respiratory distress syndrome (ARDS) (Tagami and Ong, 2018; Li and Pauluhn, 2019). Considering the variability of rats from the control groups compared (see Figs. 3 and 4), absolute lung weights were not conclusively increased in any study. As rationalized above, organ-to-body weight ratios were not considered due to long food deprivation as major confounder prior to terminal sacrifice. The multiple variables

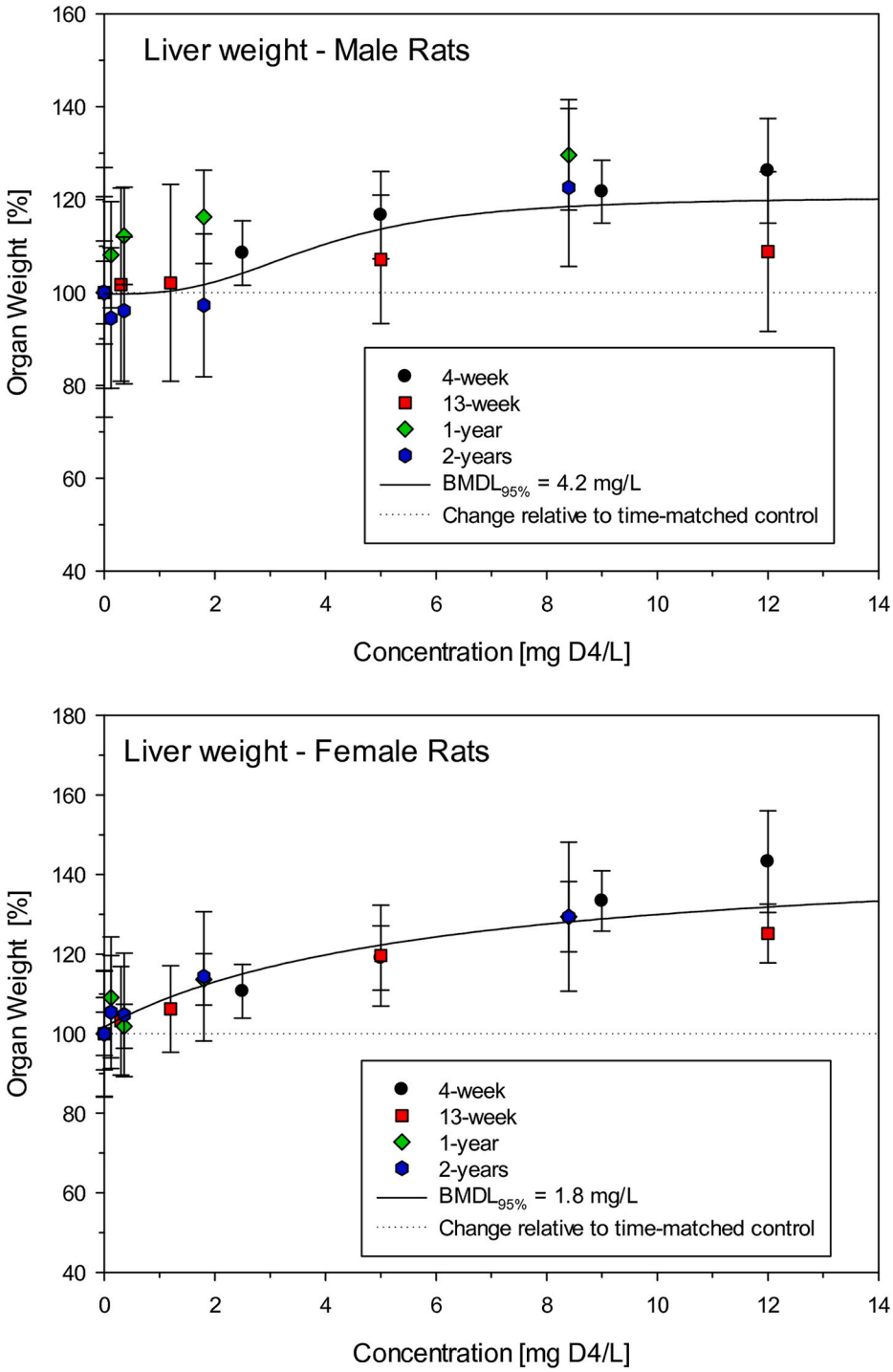

Fig. 8. Benchmark analysis of absolute liver weights from D4-exposed male (top) and female (bottom) rats covering exposure periods from 1- to 24-months. Data points represent means ± S.D. of 10–20 animals/group/sex (for details see SCCS, 2010; 2016).

associated with pre-terminal anesthesia or euthanasia may affect the perfusion of organs leading to variable discoloration of organs (Boivin et al., 2017; Reed et al., 2009). Histopathology findings were described as minimal to slight alveolar inflammation (alveolitis) in the absence of any increased lung weights seem not to fulfil the criterion "adverse". Vacuolation of the zona fasciculata of the adrenal cortex and thymic atrophy were seen in most of the animals but was most pronounced at the highest concentration. The toxicological significance of these findings appears to mirror the nociceptive response to sensory stimulation alluded to in the method section (see Pauluhn, 2018a).

3.4. Risk characterization

3.4.1. Systemic toxicity

The SCCS (2010, 2016) has identified the liver as a potential target organ following repeated-dose oral exposure and liver and lungs as potential target organs for non-cancer outcomes following repeated-dose inhalation exposure. The effects observed on the liver were mainly an increase in weight in the absence of histopathological lesions and no biologically relevant alterations in enzymatic activities. It was concluded that the reported hepatocellular hypertrophy was non-adverse.

Liver weight-to-body weight ratios and absolute liver weights from 1-, 3-, and 24-month studies on rats (6 h $\times$ d^{-1}, 5 d $week^{-1}$) exposed to D4 were normalized to their time-matched controls (100%) in Figs. 7 and 8. Growth curves of male and female rats differed. Due to the confounders detailed above, the benchmark analysis on absolute liver weights (Fig. 8) is given preference. Overall, similar to the observations made on lung weights, the increased liver weight follows a dose per day paradigm rather than any cumulative frequency of exposure-related paradigm. The time-related changes resemble a steady state-like response without time-related aggravation. This outcome fulfills the criterium for an adaptive response. The BMDL95% values on absolute liver weights of male and female rats were 4200 and 1800 mg D4/m^3, respectively. No change in liver weights was found in rats exposed to D5 and D6 (Figs. 4–6). The much lower BMDL95% values derived on portal-of-entry outcomes are believed to be entirely protective for hepatic effects.

3.4.2. Physicochemical factors and modes of action in the respiratory tract

As alluded to in the methods section, semi-volatile liquid substances nebulized as polydisperse aerosol or volatilized at temperatures producing highly supersaturated atmospheres upon cooling and dilution undergo complex coupled equilibria involving evaporation, nucleation, condensation, and coagulation. Although Vsat plays a key role in the derivation of the course taken, it must be recognized that it is defined as the partial pressure required for mass equilibrium for a flat liquid surface. The evaporation rate and lifetime of aerosol depends on multiple substance-specific (e.g., droplet size and shape, surface tension, and vapor pressure) and surrounding environmental conditions (e.g., temperature, relative humidity, and dilution rates). The Vsat's shown in Fig. 9 rely upon measurements from mass equilibria defined for a flat liquid surface whereas for the curved surface of particles, a greater partial pressure is required to maintain mass equilibrium (Hinds, 2011; Soderholm and Ferron, 1992). It could be speculated that surface tension lowering properties affect the evaporation rate of aerosol, i.e., once present in their liquid aerosol phase, evaporation rates decrease. Accordingly, a tendency of experimental artifacts can be envisaged, *viz.* the more volatile D4 aerosol might evaporate faster than the less volatile D6. It appears plausible that for D4 and D5 the major findings in both the proximal the nasal cavities and pulmonary region predominantly occur in the range and above Vsat. However, for D6 these aerosol-specific findings cannot be excluded below Vsat. Due to the high settling velocity of larger coagulated aerosol, additional high-concentration artifacts may occur near Vsat under the somewhat 'closed' conditions applied in inhalation systems. Under the thermodynamically 'open' exposure conditions experienced by humans such conditions can hardly be reproduced.

In most studies the nominal concentrations were in the range of Vsat at the top and next lower levels under the given conditions of study (Fig. 9). Thus, at concentrations high enough to poise the aerosol phase, the relative abundance of goblet cells increased at the point of initial aerosol deposition. Lung weights neither increased concentration- nor frequency of exposure-dependently (Figs. 4–6). Although residual aerosol may have interacted with pulmonary surfactant with increased AM, the alveolar barrier function and pulmonary fluid balance remained unchanged. Accordingly, despite the high concentrations used, the typical hallmarks characterizing any alveolitis or pneumonitis were absent.

3.4.3. Inhalation dosimetry model for semi-volatile substances

Basic concepts of inhalation dosimetry in risk characterization have

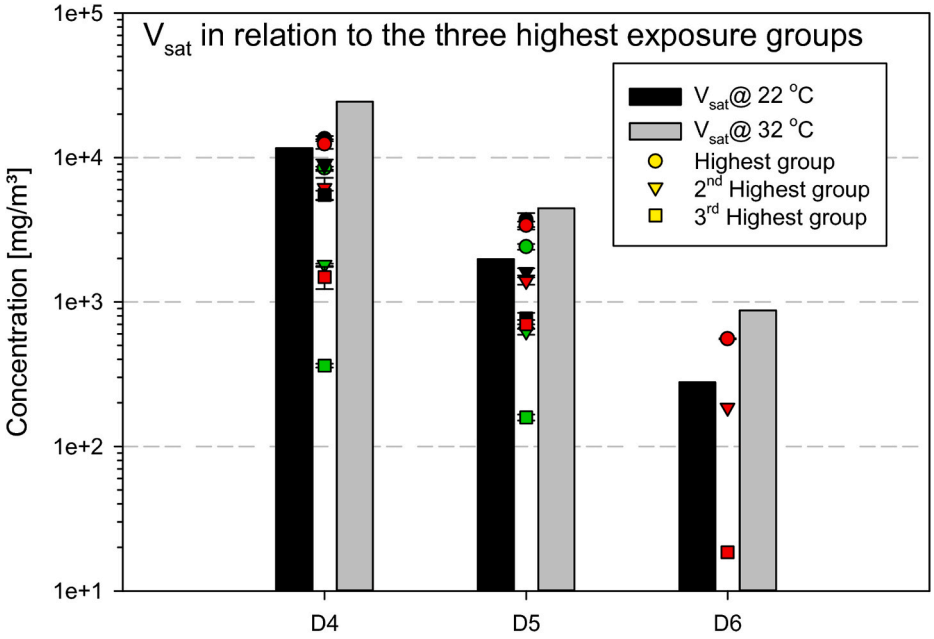

Fig. 9. Comparison of vapor saturation pressures (V_{sat}) at the levels of nostrils (22 °C) and lung (32 °C) (bars) relative to the top (circle) and the two next lower (triangle and square) nominal concentrations used in the 4-(black), 13-(red), and 104-week (green) inhalation studies. In case studies with nose-only and whole-body exposure were available, preference was given to the former. All 104-week studies used the whole-body mode of exposure. Preference to nominal concentrations – which did not appreciably differ from the actual breathing zone concentrations – were given preference because they were determined by consumed test compound every exposure day. Data points represent means ± S.D. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

been published (Kuempel et al., 2015; U.S.-EPA, 1994). These concepts use PODs from animal inhalation studies based on adverse health effect for extrapolating a human-equivalent concentration (HEC) by applying a dosimetric adjustment factor (DAF) to account for differences in the factors that influence the internal dose in each species. Additional uncertainty factors (UFs) must be applied to account for recognized uncertainties in the extrapolations from the experimental data conditions to an estimate appropriate to the assumed human scenario. The composite (UF_C) commonly considers four factors, addressing (1) sensitive humans relative to the healthy average humans, (2) differences in target organ toxicity in animals and humans, (3) extrapolation of studies of subchronic to chronic duration, and (4) the extrapolation from a $LOAEL_{HEC}$ to $NOAEL_{HEC}$. When adopting this concept for the translation of inhalation exposure studies with D4, D5, and D6, not only the concentration and inhaled C x t but also the substance-specific phase and associated phase-specific Mode of Action (MoA) prevailing under the given animal and human exposure must be appreciated as illustrated in Figs. 4–6 and 9.

To exclude the phase-specific and V_{sat}-dependent events from occurring in humans, the airborne concentration must not be exceeded under any given V_{sat} at ambient temperature (22 °C). A tenth of this threshold is taken as the generic surrogate NOAEL (= V_{sat}/10) to exclude liquid-phase events from occurring. Notably, in this context, 'A' in NOAEL stands for adaption rather than adversity. The metric at exposures in the range and above V_{sat} follows the commonly applied paradigm C x t x DAF. The DAF prevailing at V_{sat}/10 differs from that for aerosol since the inhalation of a poorly water-soluble vapor leads to steady state within short periods of time. The DAF of aerosol determining deposition, retention, and absorption changes from a C x t -proportional ventilation-dependent deposition of aerosol to a steady state determined by vapor:blood partitioning and blood perfusion. As already emphasized in the preceding sections, opposite to the aerosol phase, most of the inhaled vapor phase is exhaled unchanged.

At the Vsat/10 of D4 and D5, a proportional relationship of the 4 h-LC_{01} and the V_{sat} at 32 °C is demonstrated. However, when applying this relationship to D6 the respective V_{sat}-based predicted 4 h-LC_{01} yields 873 mg/m^3 (under the given conditions of study the Vsat was 314 mg/m^3, see Fig. 9). This estimated 4 h-adjusted LC_{01} is incompatible with the findings of the 90-day, 6 h/day, 5 days/week repeated exposure study on rats with the highest average exposure level of 30 ppm (555 mg/m^3; 4 h-adjusted C x t: 833 mg/m^3). Despite subchronic exposure, this 4 h-adjusted C x t of 833 mg/m^3 was well tolerated without treatment-related effects on clinical observations/detailed physical examinations, body weights, food consumption, hematology and clinical pathology, organ and especially lung weights, and macroscopic findings. A slight increased incidence of foamy AM with inclusions was noted at 30 ppm in female rats. During the first exposure week average daily concentrations of 33 ppm (611 mg/m^3;4 h-adjusted C x t: 916 mg D6/m^3) were recorded on 4 exposure days without any evidence of acute effects as well.

When interrelating the 4-h adjusted C x t/day of 833 mg D6/m^3, this subchronic NOAEL based on non-lethal endpoints is substantially higher than the Vsat-22 °C (278 mg/m^3). This suggests that the delivered C x t to the pulmonary region was apparently too low to cause any functional instability, supporting the hypothesis that the pool size of surfactant is large enough not to be compromised by the inhaled dose of aerosol even well-above the Vsat of D6. The following precautionary approach was taken for estimating the 4-h LC_{01} of D6: $NOAEL_{90\text{-}day}$ x 3 → $LOAEL_{acute}$ → 4-h LC_{01} = 2500 mg/m^3. As a proof-of-concept approach, the 4-h LC_{01}/Vsat-32 °C ratios for D4, D5, and D6 are compared (units: [g/m^3]/[g/m^3]) yielding 0.94 (23.0/24.35), 1.03 (4.6/4.47), and 2.9 (2.5/0.87), respectively. As already concluded earlier in this paper, the lower respiratory tract findings observed for D4 and D5 occur at a 4-h LC_{01}/Vsat-32 °C of about 1 whereas the Vsat of low-volatility cVMS (≥D6) limits the respirable C x t of aerosol to a level not exceeding the buffering capicity of the surfactant pool. Therefore, the POD based Vsat/10 appears to be a valid approach for the more volatile cVMS with molecular weights < D6 whereas for the less volatile cVMS with molecular weights ≥ D6 the Vsat/10 should be limited 100 mg/m^3. This estimate is based on the NOAEL/10 ≈ 100 mg/m^3 of the D6 subchronic study. Recalling that the described pulmonary response is caused by physical interactions of liquid-phase cVMS with liquid pulmonary surfactant, the mass metric must be given preference to any molar metric.

Published procedures for deriving human equivalent POD (POD_{HEC}) are described in detail elsewhere (Kuempel et al., 2015; U.S.-EPA, 1994). These procedures are applied to associate the adaptive responses observed in animal inhalation exposure studies to derive a health effect-based POD which then is extrapolated to a POD_{HEC} by applying inter-/intra-species dosimetric adjustments and normalizations. However, to adopt this concept to semi-volatile liquid aerosols of cVMS the following aspects need to be considered: (1) Liquid aerosol from volatile substances may evaporate or coagulate depending in their concentration relative to Vsat and surrounding temperature. Hence, any measured, welI-defined stable particle size in inhalation chambers or within the respiratory tract cannot be expected; (2) Similarly, phase-shifts with differing kinetics of deposition, retention, and absorption may occur; however, they can only be qualified as 'equilibrium shifted toward liquid aerosol' or 'equilibrium shifted toward vapor'. These conditions were believed to occur at the highest LO(adaptive)EL at the prevailing Vsat. On the other hand, any concentration equal to Vsat/10 is believed to be representative of the vapor phase; (3) This issue is complicated further because phase-specific outcomes are C-dependent relative to Vsat whereas the adaptive and potentially adverse phenomena are $C_{liquid\ aerosol}$ x t-dependent above Vsat; (4) The first step of this concept is to adjust the rat NOAEL to account for differences between the experimental regimen and the human exposure pattern (e.g., intermittent occupational vs. continuous consumer): $NO(A)EL_{ADJ}$ ≈ Vsat/10 × exposure duration per day animal:human × exposure days per week animal:humans (for details see legend of Table 1). The fractional regional deposition of liquid aerosol at Vsat (animal exposure) and Vsat/10 (human exposure) was assumed to be 1 : 0.1 which is conservative since any concentration of vapor at Vsat/10 would facilitate over-proportional vaporization of the liquid phase. Based on the more recent recommendations (ISO 18562-1, 2017; ISO 10993-17, 2002; Kuempel et al., 2015), the adjustments for exposure duration per exposure day are addressed by the species-specific ventilation (VE). Dosimetric adjustment factors (DAFs) were segregated for effects occurring in the upper (URT) and lower respiratory tract (LRT) tract. These included the phase-specific deposition/retention fraction (DF). The normalization factor NF adjusts for the difference of the surface areas of interest in humans 'H' and animals 'A'. The respective parameterization applied for the calculation the Occupational Exposure Level (OEL) or Reference Concentration (RfC) by equation (1) is detailed in Table 1.

Equation (1): $OEL \text{ or } R_fC = POD_{HEC}/UF_C = NO(A)EL_{ADJ} \times (VE_A/VE_H) \times (DF_A/DF_H) \times (NF_H/NF_A)/UF_C$

The Vsat(22 °C)/10 based $NO(A)EL_{ADJ}$ for the exposure of workers and consumer and the calculated reference concentrations for workers and consumers are detailed in Table 2. The most conservative estimate is given preference. In brief, the estimated OELs derived for the adaptive effects occurring in the URT and LRT are essentially identical (Table 2). The 8-h Workplace Environmental Exposure Levels (WEELs) derived for D4 and D5 in a more conventional way were 10 ppm (WEEL, 2014, 2015) which translates to mass concentrations for D4 and D5 of 120 and 151 mg/m^3, respectively. The respective estimates given in Table 2 are 989 and 169 mg/m^3. These differences stem from the ≈5-times lower acute toxic potency of liquid phase D4 as compared to D5. For consumers, the R_fC was lower for the LRT compared to the URT due to the LRT-specific adjustment to pre-existing ARDS-like pulmonary diseases.

The possibility of simultaneous exposures to several cVMS sharing

Table 1
Parameterization of equation (1) for portal-of-entry effects.

	Worker	Consumer
VE_A/VE_H [$m^3d^{-1}/m^3\ m^3d^{-1}$][a]	0.096/9.6 (0.01)	0.096/20 (0.005)
DF_A/DF_H - URT [%/%][b]	100/10 (10)	100/10 (10)
DF_A/DF_H - LRT [%/%][c]	30/3 (10)	10/1 (10)
NF_H/NF_A - URT [cm^2/cm^2][d]	160/6.7 (23.9)	160/6.7 (23.9)
NF_HNF_A - LRT [m^2/m^2][e]	102/0.4 (255)	70/0.4 (175)
UF_A[f]	3	3
UF_H[f] (LRT)	10	30
UF_D[f]	1	1

Equation (1): OEL or RfC = $POD_{HEC}/UF_C = NOAEL_{ADJ} \times (VE_A/VE_H) \times (DF_A/DF_H) \times (NF_H/NF_A)/UF_C$; the subscripts 'A' and 'H' stand for animals (i.e. rats) and humans, respectively; the $NOAEL_{ADJ}$ adjusts for the frequency of exposure per week of A relative to H. The subscript 'D' stands for duration as explained in footnote 'f'. For workers and consumers these factors are 1 and 5/7, respectively; the normalization factor NF adjusts for the difference of lung surface area of H and A (NF_H/NF_A). Due to the larger lung of humans relative to rats, the human lung is more robust to changes related to Laplaces' law. UF_C represents the combined UFs given in the Table. Values in brackets are calculated ratios.

[a] Respiratory ventilation (VE) per duration of exposure -rats: 6 h d^{-1}, -workers: 8 h d^{-1}, -humans: 24 h d^{-1} were 0.8, 0.29, and 0.2 L/kg-bw/min, respectively (Bide et al., 2000; Pauluhn and Thiel, 2007).

[b] Potential deposited fraction of liquid aerosol of in the nasal passages at conditions of $\geq V_{sat}$ in inhalation studies and $V_{sat}/10$ during human exposure assuming that the fraction of aerosol is linearly linked to the V_{sat}.

[c] Potential deposited fraction of liquid aerosol of D4 and D5 in the pulmonary region at conditions of $\geq V_{sat}$ in inhalation studies and $V_{sat}/10$ during human exposure assuming that the fraction of aerosol is linearly linked to the V_{sat}. Unlike the V_{sat} of D4 and D5, the lower V_{sat} of D6 has less capacity to exhaust the buffering capacity of pulmonary surfactant. The values given are arbitrarily selected but assuming that their ratio is conservative due to the longer travel time of aerosol and the approximately 1000-fold larger human lung relative to the small lung of rats.

[d] The surface area of the nasal cavity of humans is about 160 cm^2, or even 96 m^2 if the microvilli are included (Gizurarson, 2012). In rats the surface area of the squamous and respiratory epithelium is 6.7 cm^2 (Alvites et al., 2018). Other sources refer to the normalization factors $NF_H/NF_A = 200\ cm^2/15\ cm^2 = 13$ (U.S.-EPA. 1994). The more recent source was given preference.

[e] For calculation of the NF for the pulmonary region various combinations of human and rat pulmonary surface areas have been used in the literature, e.g., 62.7 m^2 for humans and 0.41 m^2 for rats (Ji and Yu, 2012), 143 m^2 for humans and 0.48 m^2 for rats, 62.7 m^2 for humans and 0.55 m^2 for rats (Yu, 1996) and 79 m^2 for human lung and 0.29 m^2 for rats (Jarabek et al., 2005). MPPD software default calculations 57 m^2 and 0.297 m^2 for humans and rats, respectively (U.S.-EPA, 2004; Fröhlich et al., 2016), while other groups indicated 102 m^2 and 0.4 m^2 as default lung surface area for humans and rats, respectively (Chen and Chen, 2016; Kuempel et al., 2015). Since lung surface area of rats can be more accurately adjusted to any degree of lung volume/capacity than in humans, this relationship remains undefined in humans. For workers, the values applied were those from Kuempel et al. (2015) whereas for the more sedentary consumer a surface area of 70 m^2 appeared to be more appropriate.

[f] To account for both differences in the nasal breathing in rats vs. oronasally breathing humans an $UF_A = 3$ is applied. For healthy workers periods of higher activity are addressed by a VE-related factor of $UF_H = 3$. LRT-related variability due to smoking results in a total $UF_H = 3 \times 3 = 10$. For the adult consumer (general population) the same UF_A as for workers is applied. Any increased VE and changes in breathing patterns either activity- or age-related is addressed by an UF_H of 3. Subpopulations with ARDS-types of chronic lung diseases at LRT level are addressed by an additional UF_H of 10. Thus, the total factor yields $UF_H = 3 \times 10 = 30$. The cVMS addressed did not show evidence of airway irritation. Additional study duration-dependent adjustments from subchronic to chronic exposures (UF_D) are omitted since outcomes were determined by the C × t per exposure day independent on the frequency of exposure.

the same physicochemical properties and associated toxicodynamics must be considered. These may cause interactions at the same target (surfactant) and affecting the cumulative risk characterization of this group of chemicals. As already suggested above, the lower Vsat of the cVMS ≥ D6 have limited capacities to attain $C_{aerosol} \times t$ levels suited to exceed the compensatory capacity of the pool of pulmonary surfactant. Hence, unlike the more volatile cVMS ≤ D5 which should be dealt with compound by compound, considerations of a grouped 'Hazard Index' could be made for the less volatile homologues. Such index uses the well-established reciprocal additivity rule dealing with the fractional contribution of each component to the overall hazard (for details see OECD, 2018).

Table 2
Derivation of Human Equivalent Concentrations for occupational exposure levels (OEL) and 24 h continuous exposures of the general public/consumer (RfC).

	D4	D5	D6
Worker – 5 days/week			
$NOAEL_{ADJ}$ [mg/m^3]	1164	198	100
OEL [mg/m^3] - URT	926	158	80
OEL [mg/m^3] - LRT	989	169	85
Consumer– 7 days/week			
$NOAEL_{ADJ}$ [mg/m^3]	831	142	71
RfC [mg/m^3] - URT	318	54	27
RfC [mg/m^3] - LRT	78	13	7

URT/LRT: Upper/lower respiratory tract. The calculated Vsat at 22 °C/32 °C are as follows (unit: mg/m^3): D4: 11637/24346; D5: 1983/4465; D6: 278/873 (see Figs. 1 and 9). As detailed in section '3.4.3 Inhalation dosimetry model for semi-volatile substances', the Vsat/10 approach is considered valid only at Vsat's high enough for titrate out the pool of pulmonary surfactant. This criterium may not necessarily be fulfilled for D6 due to the low dose inhaled (see also Legend 'c' of Table 1). The POD based Vsat/10 appears to be a valid approach for the more volatile cVMS with molecular weights < D6 whereas for the less volatile cVMS with molecular weights ≥ D6 the Vsat/10 should be limited 100 mg/m^3. This estimate is based on the NOAEL/10 ≈ 100 mg/m^3 of the D6 subchronic study.

3.4.4. *Upper respiratory tract*

Goblet cell hyperplasia and shifts in the relative abundance of specialized epithelia were the most prevalent findings in the proximal nasal cavities of rats exposed to the cVMS addressed. This prevalence increased at concentrations in the range and above Vsat as conceptualized in Fig. 9. When accounting for likelihood of aerosol to be present, the increased occurrence of adaptive responses within the nasal passages supports the conclusion that "aerosol concentration" overrules "total concentration". Concurrent with this conclusion and as already alluded to in section 2.2, the respective findings observed after subchronic exposure of rats to D6 are entirely concordant with its lower Vsat and likelihood of aerosol to be present in exposure atmospheres. The slower evaporation of aerosol following deposition in the nasal passages may explain the more pronounced tendency of D6 to cause more extensive adaptions.

Mucous lubricates the mucosal surface and forms a barrier which protects the mucosal epithelium from potentially noxious intraluminal substances. The distribution of goblet cells varies with species, airway level and disease status. Their phenotype is appropriate for contributing to first-line defense of the mucosa. Goblet cells secrete in response to neural control and stimulation, but also respond to numerous other factors causing "irritation-like stimuli" (Holmgren and Olsson, 2011; Rogers, 1994). The average number of goblet cells in humans is about 4000–7000 cells per mm^2. Especially in the obligate nasal breathing rats, goblet cell hyperplasia is a commonly observed adaptive finding.

In summary, there are many significant differences in the structural and functional anatomy of the nasal cavity of man and rats. Some of the differences may be responsible for the species-specific nasal lesions that are often observed in response to inhaled toxicants. Gross anatomical variations such as turbinate structure, folds or grooves on nasal walls, or presence or absence of accessory structures, may influence nasal airflow and species-specific uptake and deposition of inhaled material. In addition, interspecies variations in the morphological and biochemical composition and distribution of the nasal epithelium may affect the local tissue susceptibility and play a role in the development of species-

specific nasal lesions. Published evidence concludes that for human risk characterization, careful consideration must be given to the anatomical differences between a given animal model and man (Chamanza and Wright, 2015). Accordingly, rat-specific adaptive responses appear to have limited relevance to calculated PODs. Based on the given "effect constellations", the lack of robust evidence of irritation-related adversity supports this point of view.

3.4.5. Lower respiratory tract

Allometric scaling of surfactant pools across terrestrial species generally focus on the intracellular Type II cell-related pool of pro-surfactant in lamellar bodies rather than its extracellular counterpart. However, a direct interrelationship can be assumed (Wirkes et al., 2010). These authors concluded that the surfactant pool is linked to a turnover time of secreted surfactant in the range of 1–10 h. In smaller species with smaller alveolar size and greater curvature, the surface tension at the air-liquid interface increases with decreasing radius, according to Laplace's law. Likewise, the surface tension would be expected to be higher in the small alveoli of a small rodent lung than in the larger alveoli of a human lung. The evolutionary adaptation of the intracellular surfactant pool size is obtained by increasing the number of these cells with increasing body mass, but not the volume of lamellar bodies per cell. Thus, under normal conditions, the size of the intracellular surfactant pool is a relatively constant parameter (Wirkes et al., 2010). Although, this does not call for an additional pool-size related adjustment, a location-specific NF is selected to account for the species differences in the extracellular surfactant pool covering the alveolar surface area (SA). The relationship between alveolar SA and the numbers of the surfactant-producing type II pneumocytes is approximately constant across species, suggesting that function, rather than body weight, is the primary determinant of cell size (Stone et al., 1992). The terms in equation (1) determining the local kinetic dosimetric and dynamic effect-based species differences are constituted by $(VE_A/VE_H) \times (NF_H/NF_A)$. When applying the parameterization given for VE and SA in Table 1 and that published by Stone et al. (1992), this product yields for the SA-, volume- and number-based comparison of the alveolar septal components 0.84, 2.89 and 1.94, respectively. For this analysis, the most conservative SA-based approach was taken. Overall, UFs adjusting for differences in local metabolism do not apply. The dynamic differences caused by physicochemical factors within a matrix highly conserved across mammalian species render the rat more susceptible compared to humans. Thus, when applying the species-specific dosimetry adjustments for the portal-of-entry related target tissues, the residual uncertainties can be resolved by a relatively small UFc.

In summary, it is concluded that substances altering the spreading/surface tension properties of pulmonary surfactant may affect the forces maintaining alveolar homeostasis especially in rats but lesser in humans. Any outbalanced homeostasis increases the fluid-balance of the lung (see Starling's equation) which is readily quantifiable by increased absolute lung weights in rats. The pool of alveolar macrophages involved in surfactant recycling and homeostasis is kg-body weight adjusted 7-times higher in humans compared to rats, both by increased size and number (Pauluhn, 2010). Hence, some minimal histopathological adaptions serving the endocytosis and recycling of dysfunctional surfactant can be predicted at the dose levels at which liquid aerosol may gain access to the alveolar region. However, in the absence of increased absolute lung weights with the cVMS (see Figs. 4–6), this descriptive evidence cannot be equaled to be adverse. From that, all inhalation studies with the cVMS appear to be biased by an incorrect prejudice, namely that a generic adaptive non-adverse acute response is taken as adverse in the absence of chronic aggravations.

4. Conclusions

When comparing studies with different concentrations and exposures from acute to chronic, responses in the very proximal region of the respiratory tract occurred if aerosol can be predicted to be present either due to the concentration relative to Vsat or the generation principle. Nonetheless, an increased abundance of Goblet cells in the nasal passages is a common adaptive response to haptic or physicochemical stimuli in the rat. When comparing the volatility of D4, D5, and D6, the surface dose and depth of penetration of aerosol into the respiratory tract is expected to be higher for D6 (consistent with the effects confined to the nasal passages) than for its more volatile homologues (D4 and D5). In the pulmonary region, lipid-laden alveolar macrophages, often described as 'foamy' or 'vacuolized', occurred, and is entirely an adaptive response to 'deposited dose of aerosol' and the formation of complexes with surfactant rather than evidence of any substance-specific adversity. In none of the studies was there demonstrated evidence of adversities typically occurring following exposure to irritants, e.g., epithelial keratinization, degeneration of olfactory epithelium or epithelial squamous metaplasia of the laryngeal region. Although the phase-specific adaptive effects occur in the very proximal airways at lower concentrations for the less volatile cVMS, the actual hazard decreases since the likelihood of unintended exposure to aerosol decreases as well. Although the quantification of aerosol failed by the equipment available at the time of study, the hypothesis articulated in this paper was verified *post hoc* using specialized instruments (report in preparation).

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Declaration of competing interest

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