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Adverse Outcome Pathway for Antimicrobial Quaternary Ammonium Compounds

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ABSTRACT

Quaternary ammonium compounds (QACs) or quats are a large class of antimicrobial chemicals used in households and institutions as sanitizers and disinfectants. These chemicals are utilized as food processing sanitizers, algicides, in the process of water treatment, and preservatives in cosmetics. The aim of this study was to determine an Adverse Outcome Pathway (AOP) whereby two widely used QACs, alkyl dimethyl benzyl ammonium chloride (ADBAC) and didecyl dimethyl ammonium chloride (DDAC), may result in respiratory tract and gastrointestinal tract effects. When inhaled or ingested, these QACs are incorporated into the epithelial cell membrane at the point of contact. With sufficient dosage, the epithelial membrane is disrupted, reducing its fluidity, and releasing cellular contents. Further, ADBAC and DDAC might disrupt mitochondrial functions leading to decreased ATP production. Both events might lead to cell death, either attributed to direct lysis, necrosis, or apoptosis. Pro-inflammatory mediators are recruited to the tissue, inducing inflammation, edema, and excess mucus production. The primary tissue-level adverse outcome is epithelial degeneration and dysplasia. Most important, no apparent metabolism or distribution is involved in QAC action. Based upon this knowledge, it is suggested to replace default Uncertainty Factors for risk assessments with a set of Data Derived Extrapolation Factors.

KEYWORDS

Adverse Outcome Pathway; Data Derived Extrapolation Factors (DDEFs); quaternary ammonium compounds (QACs); risk assessment

Introduction

Quaternary ammonium compounds (QACs), also known as quats, are a large class of chemicals used in a variety of consumer and industrial products with unique attributes that make them an important public health tool, particularly due to their antimicrobial properties (Ogilvie et al. 2021; Zubris et al. 2017). QACs have a long history of safe use and are routinely reviewed by regulatory authorities for safety in the marketplace (Biomonitoring California 2020; ECHA 2015a; ECHA. 2015b). There are several QAC sub-classes, and sometimes these sub-classes have very different functions such as herbicidal QACs versus non-herbicidal QACs (Biomonitoring California 2020). The aim of this study was to focus on two main antimicrobial sub-classes: alkyl dimethyl benzyl ammonium chloride (ADBAC)-type QACs which includes the commonly used benzalkonium chloride and didecyl dimethyl ammonium chloride (DDAC)-type QACs. ADBAC and DDAC are groups of active ingredients registered with the U.S. Environmental Protection Agency and other global agencies for antimicrobial products.¹ QAC antimicrobial products include

household and institutional sanitizers and disinfectants, food processing sanitizers, algicides, water treatment systems, and preservatives in cosmetics (Bureš 2019; Choi et al. 2018; DeLeo et al. 2020; Piola and Grandison 2017; Zubris et al. 2017). The toxicology of these compounds has been reviewed extensively (ECHA 2015a, 2021; ECHA. 2015b; EPA 2017a, 2017b; Luz et al. 2020). In general, QACs are poorly absorbed following ingestion or dermal application and do not produce systemic toxicity, but rather cause point of contact effects. This is supported by the ECHA (2020) review of the ADBAC and DDAC database which reached the same conclusion:

“According to the available studies on toxicokinetics and metabolism as well as to the toxicity study package, no systemic effects in the absence of local effects were observed in any of those studies. Therefore, only a local risk assessment was considered necessary for the use of alkyl (C12-16) dimethylbenzyl ammonium chloride. To this aim, the local risk assessment has been performed applying the read-across principles from data presented for didecyldimethylammonium chloride (DDAC), a structurally related quaternary ammonium compound. (ECHA 2020)”.

The aim of this investigation was two-fold. First, to review and apply scientific data to outline an Adverse Outcome Pathway (AOP) whereby ADBAC and DDAC may initiate respiratory tract and gastrointestinal tract effects. Second, using the AOP and associated knowledge, to apply scientific and regulatory guidance to generate data derived extrapolation factors (DDEFs) for human health risk assessment of ADBAC and DDAC following inhalation and oral route exposure. DDEF is the term used by the U.S. Environmental Protection Agency (EPA 2014) and is similar in concept to the World Health Organization's (WHO) "chemical-specific adjustment factors" (CSAFs) (WHO 2005) and the EU REACH program's use of "informed adjustment factor" and "substance-specific adjustment factors" as defined by the European Center for Ecotoxicology and Toxicology of Chemicals (ECETOC) (ECETOC 2010). The development of DDEFs considers existing regulatory guidance on DDEFs from the EPA, the National Academy of Sciences (NAS), and EU and chemical-specific data on DDAC and ADBAC.

Individuals may be exposed to QACs orally via indirect food contact or from incidental hand-to-mouth transfer from treated surfaces (Hagi-Pavli et al. 2014; Luz et al. 2020). Inhalation of QACs might occur from aerosol formation of breathable particle sizes, household sprays or from QACs adhered to dusts as QACs are not volatile (Clausen et al. 2020; Johnson 2017; Kwon et al. 2019). Exposures from typical consumer product use are well below those expected to produce adverse effects (Osimitz and Droege 2021). Thus, it is necessary to understand that there is a threshold of QAC exposure, depending on the exposure scenario, below which there is no safety concern. Safe use instructions are provided on product labels. However, at higher exposures, direct-acting irritation might occur, and the biological pathways by which that occurs are described in this paper.

Methods

Mode of Action (MOA) and Adverse Outcome Pathway (AOP)

The Mode of Action (MOA) of a chemical or chemical class is defined as a series of biologically plausible events that lead to an effect. The World

Health Organization's International Programme on Chemical Safety (WHO IPCS) developed a framework to organize and present chemical-specific MOA information (Boobis et al. 2008; Meek et al. 2014; Seed et al. 2005; Sonich-Mullin et al. 2001). This led to development of the AOP analysis (OECD 2017, 2018) based upon the concept that toxicity results from the chemical reaching a critical target and triggering a molecular initiating event (MIE). Given sufficient dose this is followed by downstream key events (KE) leading to an adverse outcome. An understanding of the AOP may be used to develop DDEFs to replace default uncertainty factors (UF) for risk assessment (EPA 2014). These DDEFs address interspecies variability, intraspecies variability, and varying durations of exposure (short, intermediate, and long term).

The AOP scheme developed in this investigation follows principles outlined by the OECD:

"An AOP describes a sequence of events commencing with initial interaction(s) of a stressor with a biomolecule within an organism that initiates a perturbation in its biology such as MIE, which progresses through a dependent series of intermediate KEs and culminate in an adverse outcome (AO) considered relevant to risk assessment or regulatory decision-making. AOPs are typically represented sequentially, moving from one KE to another. In this respect, AOPs define a series of measurable biological changes that may be expected to occur if the perturbation is sufficiently severe in terms of potency, duration or frequency to drive the pathway all the way to the AO. Importantly, AOPs do not describe every detail of the biology but instead focus on describing critical steps or checkpoints along the path to adversity, which are both measurable and have potential predictive value. (OECD 2018)".

An AOP is presented for the adverse effects resulting from inhalation and oral exposure to QACs (Figure 1). Both oral and inhalation routes of exposure share the same MIE and several of the initial KE. Once inflammation and pro-inflammatory signaling is underway (KE-5), the AOPs diverge, and the AO become route-specific.

Molecular Initiating Event – (MIE) (inhalation and oral routes) – Incorporation of QAC in Cell Membranes

Numerous studies examined how disinfectants, including QACs, kill or inhibit unwanted microbes (Gilbert and Moore 2005; Wessels and Ingmer 2013).

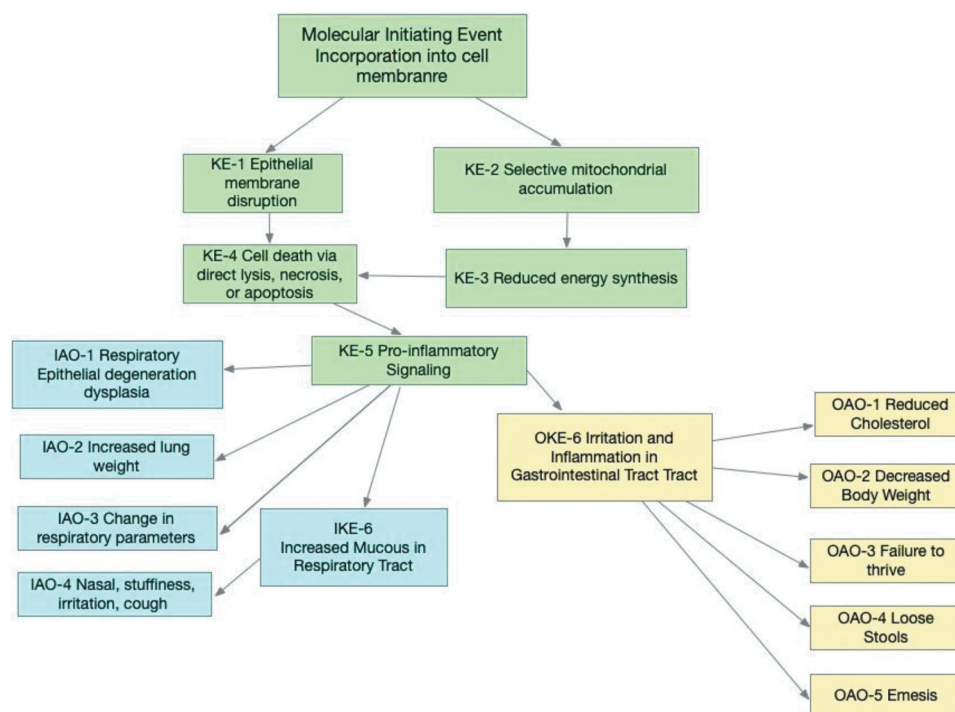


Figure 1. Adverse outcome pathway for qACs.

The MIE for QAC results from the QAC long alkyl chains' ability to "permeate into the membrane and disrupt its physical and biochemical properties" of surface cells such as epithelial cells of the eye, skin, lung or stomach (Wessels and Ingmer 2013). The charged nitrogen 'head' of the QAC remains at the membrane surface and the hydrophobic carbon chains are in the membrane bilayer. Eventually the increased concentration leads to decreased fluidity of the membrane and formation of holes in the membrane that enable the cell contents to leak out, which may further lead to cell lysis, repair or death.

Key events (KE) – inhalation and oral routes of exposure

For each KE below, biological changes are presented that occur and the scientific evidence supporting those events. Please refer to Figure 1, as some KEs are relevant for both inhalation and oral exposures while others are specific to one route of exposure.

KE-1-epithelial membrane disruption

Description of Event. Gilbert and Moore (2005) described the progression of membrane disruption by QAC molecules as these compounds accumulate in the membrane. The charged head group of the

QAC remains at the membrane surface and the carbon-chain tails align with the phospholipids. Eventually, the increased chemical concentration leads to diminished membrane fluidity and void formation, which may further result in initiation of cell lysis and repair or death mechanisms. Interaction with cell membranes can differ among QACs. DDAC has demonstrated high-affinity binding with significant initial uptake, whereas ADBAC appears to be adsorbed concentration-dependently along the lines of the Langmuir adsorption equilibrium model (Wessels and Ingmer 2013).

Supporting Data. Bleb formation (i.e., bulges) on the surface of cell membranes or membrane systems were found to occur at QAC concentrations > 50 mg/ml (Yoshimatsu and Hiyama 2007). Leakage of proteins from bacteria, or leakage of tracer dyes from liposomes both indicated that membrane integrity is altered with exposure to ADBAC or DDAC concentrations of approximately 3 mg/ml or higher (Yoshimatsu and Hiyama 2007). Cellular autolysis is initiated upon a breakdown in RNA and membranes, in direct relationship with potassium depletion and leakage of proteins and β -galactosidase. The overall protein level and lactate dehydrogenase (LDH) activity measured in the

bronchiolar-alveolar lavage fluid (BAL) may reflect disruption of the epithelial barrier and vascular permeability due to direct damage of airway and alveolar epithelial cells (Ohnuma et al. 2011). QAC exposure promotes what appears to be a biphasic response to cell injury with the initial effect attributed to the actual interaction of QAC with the cell membrane and, a greater effect resulting later because of the upregulated inflammatory response (Berg, Lie, and Steinsvåg 1994; Ohnuma et al. 2011).

Experiments demonstrating epithelial membrane disruption were generally conducted in either bacterial cell cultures found in gut flora (Gilbert and Moore 2005) via intra-tracheal instillation (Ohnuma et al. 2011) or other surface epithelial cells (Walters et al. 2012). These data demonstrated the surfactant effect of QAC on relevant epithelial cells, which are consistent with microscopic observations of epithelium irritation of the GI tract in dogs administered subchronic oral doses of DDAC (Cox and Bailey 1975) or rats treated with subchronic inhalation doses of DDAC (Kim, Lee, and Lim 2017). KE-1 was also detected in cultured human respiratory mucosa (Berg, Henriksen, and Steinsvåg 1995) and in bacteria found in human gut flora (Wessels and Ingmer 2013).

KE-2-selective mitochondrial accumulation

Description of Event. Lipophilic cations including QACs were reported to selectively accumulate in mitochondria (Inácio et al. 2013; Liberman et al. 1969; Ross et al. 2005). This selective accumulation is driven by a membrane potential-dependent mechanism as shown by Bragadin and Dell'Antone (1996). It was postulated that the negative charge of the mitochondrial matrix coupled with the QAC positive charge and ability to pass through biological membranes enables compounds to accumulate in mitochondria several 100-fold above intracellular levels (Murphy and Smith 2007). Because mitochondria are absent in prokaryotic cells such as bacteria, the discussion of this KE and subsequent downstream events and effects are limited to mammalian cells.

Supporting Data. Inácio et al. (2013) demonstrated that mitochondrial membrane function was disrupted in Madin-Darby canine kidney (MDCK) II cells at concentrations below which produced cell lysis. QACs require time to build up

concentrations in the mitochondria sufficient to initiate effects such as cytokine release measured 3 hr after treatment (Inácio et al. 2013). In several studies KE-2 follows a dose-response relationship in that increased QAC or other cationic surfactant result in elevated presence in the mitochondria and/or loss of mitochondrial function as measured by barrier function, ATP production, and loss of membrane potential (Bragadin and Dell'Antone 1996; Inácio et al. 2013; Ross et al. 2005).

Numerous models in both animal and cellular systems such as, isolated mitochondria spanning multiple mammalian species including rodent, human and dog and methods to determine mitochondrial function demonstrated results consistent with proposed mechanism. Inácio et al. (2011) investigated nonionic, zwitterionic, anionic, and cationic surfactants and found that cationic surfactants were the only group in which cytotoxicity occurred at concentrations too low to provoke cell lysis (Inácio et al. 2011). Data suggest that the positive charge is central to enhanced cytotoxicity that differentiates cationic surfactants from other surfactants cytotoxic through a less specific surface-active action.

Kwon et al. (2014) noted effects for KE-2 in human cell models. Although substantial differences are known between cell membrane structure and mitochondria between species, the lipid bilayer (permeable to QAC) and negatively charged mitochondria are consistent between humans and the experimental models used.

KE-3-reduced energy synthesis

Description of Event. Accumulation of QAC in the mitochondria of epithelial cells results in decreased ATP production, which leads to apoptotic or necrotic cell death (Figure 1). QACs inhibit mitochondrial complex I (Datta et al. 2017; Inácio et al. 2013) that reduces NADH and translocates 4 protons from the mitochondrial matrix to the intermembrane space, which is a primary driver of the electrochemical gradient.

Supporting Data. ATP synthase requires the electrochemical proton gradient to function. Inhibition of complex I might result in collapse of mitochondrial membrane potential observed by Datta et al. (2017), Inácio et al. (2013), and Bragadin and Dell'Antone (1996). Bragadin and

Dell'Antone (1996) proposed that the loss in mitochondrial membrane potential was mediated by increased membrane permeability as evidenced by calcium and potassium ion leakage. However, arrested complex I loss of membrane potential, due to loss of proton translocation, might also lead to ion release from losing the electrochemical gradient. Data suggest that complex I inhibition is likely the culprit for reduced ATP production, but mitochondrial membrane disruption is a plausible alternative or complimentary mechanism that might induce similar effects on energy production. Changes in mitochondrial morphology observed by Inácio et al. (2013) might result from membrane disruption, but observed fragmentation was also associated with apoptosis. Finally, Datta et al. (2017) screened several compounds and demonstrated similar dose responses between cetylpyridinium chloride and ADBAC in reducing ATP production and oxygen consumption. Evidence indicates that the approach here to generalize cetylpyridinium chloride (Datta et al. 2017) and cetrimonium bromide (Bragadin and Dell'Antone 1996; Inácio et al. 2013) behavior to that of the ADBAC and DDAC is appropriate when discussing mitochondrial effects.

KE-3 consistently follows a dose-response relationship whereby QAC or other cationic surfactants result in decreased ATP production and threshold-based loss of membrane potential in a dose-dependent manner. Datta et al. (2017) showed increased benzalkonium chloride concentrations produced diminished oxygen consumption and ATP production. It is important to note that there appears to be a threshold concentration where benzalkonium chloride does not significantly impact ATP production or mitochondrial oxygen consumption compared to negative control.

Numerous models animal and cellular such as isolated mitochondria spanning multiple mammalian species including rodent, human and canine and methods to assess mitochondrial functions show results consistent with the KE-3. QACs are capable of passing through intact cell membrane and accumulate in mitochondria in polarized epithelium in human cells. Assuming that QACs reach the target tissue such as the respiratory or GI

epithelium at sufficient concentration, this effect on mitochondria is relevant to the observed QAC toxicity.

KE-4-cell death via apoptosis, direct lysis, or necrosis

Description of Event. It is well established that direct membrane lysis or decrease or depletion of cellular ATP produces cell death by apoptosis or necrosis (Eguchi, Shimizu, and Tsujimoto 1997; Inácio et al. 2011). Inácio et al. (2013) demonstrated that QACs including ADBAC produce dose-related necrotic or apoptotic cell death depending upon the ATP/ADP ratio.

Supporting Data. Inácio et al. (2011), using human cell lines, demonstrated that, at lower doses, pre-apoptotic signaling occurred whereas, high levels of QAC rapidly depleted ATP and necrotic cell death occurred. As concentrations at the cell approach the critical micelle concentration, the surface-active action of QAC predominates and results in cell lysis. Inácio et al. (2011) also examined various lengths of cetrimonium bromide, cetylpyridinium bromide, and benzalkonium bromide and found that when the hydrocarbon chain lengths were comparable, the critical micelle concentration normalized relative toxicity scale was C_{12} benzalkonium bromide $\approx C_{12}$ cetylpyridinium bromide $\gg C_{12}$ cetrimonium bromide.

KE-5-pro-inflammatory signaling and inflammation

Most of the evidence to support this KE originates from studies of the responses of respiratory epithelium. Nonetheless, it is reasonable to assume qualitatively similar responses would also occur in gastrointestinal epithelium.

Description of Event. Pro-inflammatory signaling occurs following damage-induced loss of membrane integrity leading to potential enhanced vascular permeability and protein leakage and further propagation of the inflammatory signaling cascade. This is predominantly due to cytotoxicity that includes generation of reactive-oxygen species (ROS). Cell death via apoptosis might also lead to inflammation, but likely less extensive than via necrosis. Although proinflammatory components are released from necrotic cells, this proinflammatory signal is quenched over time as the “debris” is degraded and diluted (Rock and Kono 2008). Thus,

low-level cellular injury results in limited inflammation, which is reversible and finite below a certain dose.

Supporting Data. Reactive-oxygen species and inflammation signaling was reported after ischemia-reperfusion injury (Rock and Kono 2008). Generation of oxygen or nitrogen radicals lead to activation of antioxidant response elements (AREs) and pro-inflammatory gene transcription and expression including hemoxygenase-1,² and various cytokines/chemokines.

DDAC administration to the respiratory tract cells *in vivo* resulted in loss of membrane integrity enabling enhanced vascular permeability and leakage of proteins (Ohnuma et al. 2011). This led to induction of oxidative damage with evidence of detectable hemoxygenase-1 and upregulation of cytokines (Ohnuma et al. 2011, 2010; Ohnuma-Koyama et al. 2013).

Inhalation Route Specific Key Event – $KE_{inhalation-6}$ – Irritation and Inflammation in the Respiratory Tract

Description of Event. Following inhalation exposures to QACs, proinflammatory signaling induced by cell death gives rise to increased inflammatory cell count in bronchoalveolar lavage fluid, in particular polymorphonuclear cells and macrophages.

Supporting Data. In some studies, an increase in neutrophilic inflammation was observed, in a pattern simulating ozone exposure (Larsen, Verder, and Nielsen 2012). The recruitment of inflammatory mediators increased in degree and severity with dose. Based upon elevated numbers of macrophages and polymorphonuclear neutrophils in a dose-dependent manner (Larsen, Verder, and Nielsen 2012), data suggest that direct epithelial interaction with increasing concentrations of ADBAC and other QACs initiate pro-inflammatory signaling leading to inflammatory cell recruitment. Inhalation studies of ADBAC or DDAC in mice or rats showed significant elevation in inflammatory markers in bronchoalveolar lavage (BAL) fluid from short-term high doses (Larsen, Verder, and Nielsen 2012; Swiercz et al. 2013, 2008). Longer term studies (2-, 4- and 13-week studies) at lower doses produced no or small changes in BAL fluid proinflammatory markers

(Kim, Lee, and Lim 2017; Lim and Chung 2014; Weinberg 2011). A dose-dependent, threshold-based response was demonstrated.

Inflammatory mediators that recruit leukocytes also result in vasodilation and increased vascular permeability (Scallan, Huxley, and Korthuis 2010). These, and other secondary effects including protein infiltration into tissue, resulting in accumulation of extracellular fluid. Several investigators noted that QAC-mediated tissue injury from inhalation or intratracheal/nasal administration also induced edematous changes (Cho et al. 2000; Kuboyama, Suzuki, and Hara 1997; Ohnuma-Koyama et al. 2013). Elevated protein production, likely the result of cellular influx and potential neutralization of lung surfactants, was measured in BAL fluid of BAC-exposed rats (Larsen, Verder, and Nielsen 2012). Given sufficient exposure, influx of fluid from capillary leak syndrome resulting from elevated protein levels and LDH activity release was measured in BAL fluid (Ohnuma et al. 2011). The temporal relationship between QAC exposure and inflammatory response in mice (Ohnuma et al. 2011) is biphasic with an initial effect attributed to epithelial degeneration and a greater effect resulting later because of cell membrane disruption and upregulated inflammatory response.

An alternative minor pathway is the cholinergic action QACs exert on muscarinic receptors in the peripheral and central neural receptors leading to histamine release in rodent mast cells *in vitro* (Miszkiel et al. 1988b). However, H1-histamine receptor antagonists induce only a minor decrease in histamine release, suggesting this is likely a minor, pathway (Miszkiel, Beasley, and Holgate 1988a; Miszkiel et al. 1988b).

Inhalation route specific key event – $ke_{inhalation-7}$ – increased mucus

Description of Event. Nasal epithelial cells constantly produce mucus for protection and particulate transport. Mucociliary clearance aids in the removal of noxious substances that might get trapped in the mucus blanket (Stanley et al. 1985). A common response to inhaled irritants or allergens is increased mucus production (Cho et al. 2000).

Supporting Data. This irritant response accompanies clinical and histopathological observations in rats and human inhalation studies of ADBAC or DDAC. Inhalation studies of DDAC in rats for 28 days found mucus production upon histopathological investigation (Weinberg 2011). Nasal administration of BAC to rats produced congestion (Cho et al. 2000; Kuboyama, Suzuki, and Hara 1997). Nasal spray delivery of BAC in human volunteers induced mucosal swelling and discharge (Graf, Enerdal, and Hallén 1999; Riechelmann et al. 2004). Nasal congestion resulted from BAC-containing nasal spray in healthy volunteers but repeat applications over 10 days did not enhance symptoms (Storaas et al. 2000).

Inflammatory mediators and cytokines that contribute to edema and mucus production resulting from QAC insult may contribute to both downstream effects. It is reasonable to assume that QAC mediated inflammatory signaling might result in downstream mucus production consistent with the understanding of mucus secretion inducers such as inhaled irritants (Rogers 2007).

Oral Route Specific Key Event – KE_{oral-6} – Irritation and Inflammation in the Gastrointestinal Tract

Description of Event. Standard repeat-dose studies were conducted in mice, rats, rabbits and dogs via oral, dermal and inhalation routes along with developmental and reproductive toxicity evaluations (Luz et al. 2020). The results from these studies reveal a consistent pattern: local irritation rapidly followed by signs of generalized toxicity, as expected with the MOA of an irritant.

Supporting Data. Animal studies employing oral dosing resulted in inflammation of tissues lining the GI tract. Cox and Bailey (1975) treated dogs with high doses of DDAC (50 mg/kg/day) in the diet for 90 days and reported histopathological observations of slight-to-mild mucosal congestion of the small intestine, and to a lesser extent the large intestine. Further Van Miller (1988b) noted that Sprague-Dawley rats treated with 3000 ppm DDAC in the diet for 90 days initiated irritation of the gastric mucosa and/or gastrointestinal lesions; these lesions were not detected in animals given diets containing 1000 ppm.

Adverse outcomes – inhalation route

$AO_{inhalation-1}$ -epithelial degeneration, dysplasia

Description of Outcome. Animal inhalation studies demonstrated that QACs result in direct portal-of-entry tissue damage (Luz et al. 2020; Weinberg 2011).

Supporting Data. Micropipette administration of benzalkonium chloride to the nostrils of rats resulted in squamous metaplasia, degeneration of the trilaminar membrane, and mitochondrial swelling (Lebe et al. 2004). Cüreoglu et al. (2002) sprayed benzalkonium chloride into rabbit nostrils and noted mitochondrial effects and degenerative changes in the supportive and olfactory cells (Cüreoglu et al. 2002). Cho et al. (2000) performed micropipette administration of benzalkonium chloride to rats and found nasal lesions, including intraepithelial glandular formation, inflammatory cell infiltration, vascular hyperplasia, and edematous change (Cho et al. 2000). Kuboyama, Suzuki, and Hara (1997) administered benzalkonium chloride nasally via micropipette to rats and noted edema, desquamation, and inflammation. Data demonstrated a dose response, including a no effect threshold where increased BAC concentration resulted in elevated epithelial desquamation. Ohnuma-Koyama et al. (2013) showed that intratracheal instillation of DDAC to mice produced inflammation, inflammatory cell infiltration, edema, and cell death. Choi et al. (2020) demonstrated that acute inhalation exposure to 20 mg/m³ f benzalkonium chloride was associated with deep breathing and nasal discharge as well as histopathological alterations in the nasal cavity and lungs of Fischer-344 rats postulated to be due to oxidative damage. Although no direct human data are available demonstrating this effect, it is biologically plausible. QACs are irritating and, other than accidental overexposure, it is unlikely that humans would be subjected to sufficiently high enough concentrations of QACs to elicit such damage.

It is clear from the studies above that sufficient necrotic or apoptotic cell death initiates a tissue level effect. Mitochondrial effects noted by Lebe et al. (2004) and Cüreoglu et al. (2002) further support the link between mitochondrial dysfunction and ultimate epithelial degeneration. Overall, the portal-of-entry effects were relatively consistent

in their dose response and composition: inflammatory cell infiltration, membrane disruption, mitochondrial degeneration, edema, and inflammation were commonly noted across most investigations.

AO_{inhalation}-2-increased lung weight

Description of Outcome. Some inhalation toxicology studies in animals demonstrate that QACs might produce an increase in lung weights as determined at necropsy.

Supporting Data. Rats administered aerosols of ADBAC following short-term (< 7 days), high-dose inhalation exposures (Swiercz et al. 2013), or of DDAC (Kim, Lee, and Lim 2017; Lim and Chung 2014; Swiercz et al. 2013; Weinberg 2011) following lower-dose, longer-term exposures (14–90 days) resulted in increased lung weights. Acute inflammation and/or evidence of edema were observed accompanied by elevated lung weights. However, a longer duration of dosing did not result in a rise in lung weights which is consistent with an irritant response where lower doses are tolerated and do not progress. This is also observed with other point-of-contact irritant responses.

AO_{inhalation}-3-reduced tidal volume and other changes to respiratory parameters

Description of Outcome. Reduced tidal volume and other respiratory parameters were reported in rats and mice (Larsen, Verder, and Nielsen 2012; Yang et al. 2012) and humans with asthma (Miszkiel, Beasley, and Holgate 1988a; Miszkiel et al. 1988b) following short-term exposure to BAC.

Supporting Data. Irritant responses are well known to affect respiratory parameters (ECETOC 2003, 2010; Larsen, Verder, and Nielsen 2012). DDAC and ADBAC are well established direct-acting irritants, with adverse respiratory effects seen in humans and animals. Reduced tidal volume and other respiratory parameters were noted in rats and mice (Larsen, Verder, and Nielsen 2012; Yang et al. 2012) and humans with asthma (Miszkiel, Beasley, and Holgate 1988a; Miszkiel et al. 1988b) following short-term exposure to BAC. Dose-response relationships were reported in mice following short term exposure to BAC, and three other QACs (hexadecyl trimethyl ammonium bromide, hexadecyl pyridinium chloride, and dimethyl dioctadecyl ammonium bromide) (Larsen, Verder, and Nielsen 2012). Concentration-

dependent increases in respiratory frequency were observed above 1 mg/m³. Concentration-dependent decreases in tidal volume were also observed, with the highest doses producing approximately 30% reduction from baseline. Markers of the sensory irritation response indicated that QACs initiated no activation of trigeminal nerve endings, suggesting that the respiratory responses are due to irritation. Changes in respiratory parameters in rats administered aerosols of DDAC found only effects at the highest dose given (Yang et al. 2012).

AO_{inhalation}-4-nasal stuffiness, irritation, cough

Description of Outcome. Inhalation studies of DDAC in rats for 28 days noted mucus production upon histopathological investigation, with clinical signs of rales and yellow discharge reported at the highest dose (Weinberg 2011).

Supporting Data. BAC administered nasally to humans produced immediate irritation, followed later by swelling and stuffiness. Nasal administration of an ADBAC to rats produced dose-related sneezing and nasal rubbing and nasal sounds, indicating irritation and congestion (Cho et al. 2000; Kuboyama, Suzuki, and Hara 1997). The findings in animals are consistent with data in humans where nasal spray delivery of an ADBAC induced irritation, pain, mucosal swelling and stuffiness (Graf, Enerdal, and Hallén 1999; Riechelmann et al. 2004; Storaas et al. 2000). Reduced tidal volume in mice and bronchoconstriction in human subjects with asthma following higher QAC exposures were rapid, dose-related, and not observed in concurrent controls. These observed adverse outcomes are consistent with other direct-acting irritant chemicals. Dose-related and temporally related responses noted for DDAC and ABDAC follow postulated AOP. These responses are consistent for animals and humans.

Adverse outcomes – oral route

AO_{oral}-1 reduced serum cholesterol levels

Description of Effect. High doses of QACs may produce a reduction in circulating serum cholesterol levels in animals.

Supporting Data. Evidence for an effect of QACs on serum cholesterol levels is extremely limited. Nonetheless, in the interest of completeness, it is being included as a possibility as reported by

Hrubec et al. (2021) who noted in humans that QACs in blood were associated with disruption of cholesterol synthesis, increased inflammation, and reduced mitochondrial function. Serum cholesterol was determined in chronic rat toxicity studies with ADBAC (Gill, Chun, and Wagner 1991) and DDAC (Gill, Hermansky, and Wagner 1991). No significant treatment related effects were noted. Similarly, a 1-year chronic toxicity study in dogs with DDAC showed no marked effects. The *only* treatment-related observation of changes in cholesterol were noted in a 1-year chronic toxicity study in dogs with ADBAC (Goldenthal 1994). A significant decrease in serum cholesterol was noted in females of the high dose group (1200 ppm, n = 4 animals). No other clinical chemistry or organ-specific changes were detected.

Changes in serum cholesterol may constitute effects secondary to reduced caloric intake and general stress. It is notable that approximately half of the cholesterol in the human body derives from biosynthesis *de novo*. The liver accounts for approximately 10% and the intestines for approximately 10% of the amount produced each day (The Medical Biochemistry Page 2021). The remainder is a result of ingestion and GI uptake of cholesterol contained in foods. Thus, it is reasonable that damage to the GI tract due to the irritating effects of QACs may exert a significant effect on GI biosynthesis and/or uptake of cholesterol into the body, thereby reducing serum cholesterol levels. The above notwithstanding, a 1-year chronic toxicity study in dogs exposed to ADBAC indicated no consistent relationship between body weight, body weight gain, or food consumption and serum cholesterol levels (Goldenthal 1994).

Note: In Figure 1 a dashed line was used to indicate a qualitatively possible direct relationship between selective mitochondrial accumulation (KE-1) and cholesterol levels. Recently, investigators examined the effect of QACs on various steps in cholesterol biosynthesis and associated gene expression (Herron et al. 2016; Seguin et al. 2019). An exploratory *in vivo* study reported the effect of QAC on lipid metabolism-related genes in the brains of neonatal mice exposed during *in utero* to 120 mg/kg QAC (Herron et al. 2019). Unfortunately, only a single dose level was studied, a level at which Melin et al. (2014) reported clear

evidence of significant toxicity consisting of “clinical signs initiating euthanasia included: partial closure of eyelids (squinting), reduced activity, ataxia, kyphosis, hypothermia, rapid breathing and dyspnea, and cyanosis” (Melin et al. 2014).

These doses are far higher than the mean daily U.S. exposure of the population. For example, 120 mg/kg/day is almost 4000-fold as high as the average daily U.S. exposure to ADBAC and DDAC combined (0.0303 mg/kg/day) (EPA 2017a, 2017b). Thus, although a possible link in our AOP was included, this extremely high dose is quantitatively irrelevant for safety assessment of QAC for humans.

Generalized Gastrointestinal Effects (AO_{oral}-2 Failure to Thrive, AO_{oral}-3 Loose Stools, AO_{oral}-4 Emesis)

Description of Effects. The most common GI effects including decreased body weight, body weight gain, and food consumption were observed in dogs, Guinea pigs, mice and rats (Luz et al. 2020). Emesis³ was also noted following dosing in a 1-year chronic dog study (Schulze 1991).

Supporting Data. Gastrointestinal effects are observed after repeated dosing. A longer duration of dosing did not result in greater effects. This is consistent with an irritant response where lower doses are tolerated and do not progress upon repeated exposure. Decreased body weight, failure to thrive, loose stools, and emesis (in dogs) are consistent observations from oral toxicity studies.

Results

Assessment of the postulated aop for QACs

Many KEs are common to both inhalation and oral exposures (Figure 1). Following oral exposure, QACs might affect GI tract cells directly or produce GI irritation. This leads to loss of appetite, emesis, loose stools and, with long-term high doses, decreased body weight and failure to thrive. The postulated AOP for the inhalation route is well supported by *in vitro* data of lung and nasal epithelial cells, and *in vivo* studies in animals following inhalation or nasal delivery. Studies of QAC compounds in human cells and human volunteers provide a strong, clear and consistent evidence base. Sufficient dose-response, temporal data, consistent and plausible information are available in animals and humans to

substantiate the KEs depicted in the AOP scheme. For oral exposure, an extensive database including short-term and long-term oral toxicity studies in mice, rats, rabbits, and dogs consistently demonstrate that toxicity is limited to effects at the point of contact (Luz et al. 2020). Studies in which histopathology was performed presented no marked evidence of cellular toxicity in distant tissues. Changes in body weight were noted in some investigations, but these were attributed to effects of irritation and inflammation at the point of contact and reduced food consumption. Further, QACs are poorly absorbed following ingestion or dermal application and do not lead to systemic toxicity.

Uncertainties, inconsistencies, and data gaps

Evidence of ADBAC- or DDAC-induced cell death arises from *in vitro* studies using human and animal cells and from histopathology evidence from *in vivo* investigations. Although direct evidence of cell death in humans is not available, sufficient information from human and animal cell studies *in vitro* and animal *in vivo* studies show that similar inflammatory processes occur in animals and humans.

Alternate AOP for oral route of exposure – alteration of gut microflora

In sub-chronic and chronic dietary studies, QACs irritate the GI tract, but speculation is that QACs may also alter the microflora because of their potent antimicrobial properties and thus produce secondary effects of weight loss and a general decline in health via this mechanism (Cox and Bailey 1975; Miller 1988b; Osheroff 1990; Van Miller 1988a). Although no definitive data were located for this alternative MOA, it is plausible and cannot be excluded as a significant contributor to GI irritation.

Alternate AOP for inhalation route of exposure – fibrosis

Ohnuma-Koyama et al. (2013) used RNA expression analysis and immunohistochemical staining of lung cells to demonstrate that fibrotic markers were elevated from inter-tracheal exposure to DDAC. These investigators concluded that DDAC may exhibit the potential to induce fibrotic

pathways but noted that longer-term dosing studies were needed to determine if this hypothesis is accurate. However, longer-term (4- and 13-week) inhalation studies using DDAC detected no fibrotic lesions (Kim, Lee, and Lim 2017; Weinberg 2011).

Discussion

Implications for risk assessment

Regulatory agencies have generally regarded the various apparently systemic effects following oral dosing as being secondary to effects on the GI tract. For example, ECHA says with respect to ADBAC:

“The results from the studies reveal a pattern of response (local irritation/corrosion followed by reduced food intake and reduction in body weight and body weight gain) that is consistent with the mode of action of a corrosive substance. Therefore, the systemic effects observed in these studies are regarded as secondary to the local irritation/corrosion caused by the test substance and as a result no adverse systemic effects were identified and no systemic risk characterization is required. (ECHA 2015a)”

Similarly for DDAC,

“As with acute exposure, DDAC repeated intake results in death in rodents at a concentration that affects the gastrointestinal mucosa or microflora or in unspecific toxicity with the major effects likely associated to the concentration dependent cytotoxicity, irritation and corrosivity at the site of contact. Indeed, the effects on which the NOEL [sic] derivation could have been based, independently on the species tested, were the reduction in body weight and body weight gain, consistent with decreased food consumption (US ISC; EQC). It was concluded that all effects could be attributed to local gastrointestinal irritation/ corrosion and consequent reduced food intake without observing any primary systemic effect. Therefore, the derivation of a NOAEL for systemic effects was deemed inappropriate. (ECHA. 2015b)”

The same principles apply to inhalation exposures. Hence, this analysis focuses on risk assessment for local effects.

Derivation of data derived extrapolation factors (ddefs) for local effects

In risk assessment practice, UFs are applied to the toxicity point of departure (PoD) (e.g., No Observed Adverse Effect Level – NOAEL) to

account for variabilities and uncertainties in determining a health-protective toxicity benchmark. UFs for differences and intraspecies variability consist of two components: (1) a toxicokinetic component (variability in dose) and (2) a toxicodynamic component (variability in the response). The UFs for toxicokinetics encompass differences in metabolism and distribution of the chemical, whereas the UFs for toxicodynamics encompass the range of tissue responses/sensitivities to a given dose. Using the extensive database available for the QACs and guidance from regulatory agencies, DDEFs were determined to account for interspecies extrapolation, intraspecies extrapolation, and study duration.

Interspecies extrapolation

Various agencies and authoritative bodies including the European Center for Ecotoxicology and Toxicology of Chemicals (ECETOC), the U.S. Environmental Protection Agency (EPA), and the U.S. National Academy of Sciences' National Research Council (NRC) have considered the MOAs and AOPs of chemicals causing point of contact toxicity (ECETOC 2003, 2010; EPA 2014; NRC 2001; WHO 2005). EU regulators stated that sufficient evidence exists that humans are *less* responsive to irritants than animals. Examples of precedents in which DDEFs were used for specific direct acting chemicals include:

- ECETOC (ECETOC 2003) – For chemicals, such as irritants producing direct local effects, they recommended UF = 1X for both toxicokinetic and pharmacodynamic differences.
- NRC (NRC 2001) – Their Standard Operating Procedures for development of Acute Exposure Guideline Levels (AEGs) stated that when the scientific evidence indicates that the mechanism or mode of action is not expected to differ among species, such as with chemicals causing local effects, an interspecies AF of 3 is generally used.
- EPA (EPA 2017a, 2017b) – As part of their assessment of mineral acids, they stated that “... the TD component of the UF can be set to 1, if the mechanism or mode of action, such as direct-acting irritation or alkylation, and is not expected to differ significantly between species.”

Similarly for toxicokinetics they recommend a 1X UF for extrapolating from primate (source of mineral acid data) to humans.

- EPA (EPA 2021) – In their draft risk assessment for chlorothalonil, a direct acting irritant with toxicity occurring at the point of contact in the respiratory tract, they concluded that the absorption, distribution, metabolism, and excretion characteristics are not likely to have a significant effect on the response among the human population. As such, the toxicokinetic component of the interspecies extrapolation UF was reduced to 1X.

The available data and information from computer-derived models of the respiratory tract in humans and rodents indicate that local effects of water soluble gases and vapors observed in the rat nasal cavity when extrapolated to the human situation are likely to over-estimate the effects in humans by factors of at least 2–4 (ECETOC 2010). Considering this, it is conceivable that a DDEF = 1 is appropriate for inhalation of QACs.

For oral exposures to QACs, the ECHA registration dossier for benzyl-C12-14-alkyldimethylammonium chlorides that underwent comprehensive ECHA review states that considerable species-related anatomical differences are known in the stomach and remaining GI tract and that this might lead to species-related differences in response. On this basis an interspecies UF = 2X is recommended (ECHA 2021). Evidence indicates that a DDEF = 1 to 3 is appropriate for assessment of oral intake of QACs, whereas a value of 3 may be most appropriate given the limited data available on the alteration of microflora.

Intraspecies extrapolation

Since no reproductive or developmental toxicity was seen in guideline studies on ADBAC and DDAC, the EPA determined that no additional safety factor for juveniles (also known as the FQPA factor) is required (EPA 2017a, 2017b).

Further, because no toxicokinetics, i.e., distribution and metabolism (often sources of inter-individual variability) are involved in QAC adverse outcomes in either the GI or respiratory tract, and variability in response may be narrow in DDAC and ADBAC studies, an inter-individual DDEF of 3 is appropriate for both routes per existing guidance

(EPA 2017a,b; NRC 2001). Similarly, ECETOC (2010) used a DDEF of 3 for workers and 5 for general population. The NRC states that “In those cases in which the mode or mechanism of action is such that the response elicited by exposure to the chemical by different subpopulations is unlikely to differ, an intraspecies UF of 3-fold is generally used. Typically, this response involves a direct-acting mechanism of toxicity in which metabolic or physiologic differences are unlikely to play a major role.” (NRC 2001). Similar guidance is provided elsewhere (EPA 2017a, b; ECETOC 2010; NRC 2001).

Study duration

Our analysis confirms that, regardless of exposure route, QACs are point-of-contact toxicants that disrupt cell membranes that may lead to irritation or cell lysis. Consistent with this is the reversibility of irritation and downstream effects from repeated low oral or inhalation exposures of ADBAC and DDAC. The adverse effects of QACs do not involve *in situ* or systemic metabolism. Inflammatory processes are balanced with anti-inflammatory processes and mechanism of cell repair (Wilson and Wynn 2009). Thus, at low doses, epithelial cells of either the respiratory or GI tract tolerate repeated low QAC exposures without measurable effect. Consequently, short-term and long-term exposures to small doses of QACs exert similar effects and display similar NOAEL values. Given the reversibility of the inhalation-induced KE and AO (Kim, Lee, and Lim 2017; Ohnuma et al. 2011; Weinberg 2011), the reversibility of other irritants (ECETOC 2010), and balance of inflammatory and anti-inflammatory processes in the lung (Wilson and Wynn 2009), no UF is needed to extrapolate from shorter to longer term studies. This is consistent with the observations of Shusterman, Matovinovic, and Salmon (2006) that human respiratory irritation does not follow Haber's law. Data on human inhalation exposures to direct acting irritants were dependent upon concentration but minimally on duration of exposure for initiating sensory irritation.

Summary

Based upon the above discussion, Table 1 shows the scientifically supported DDEFs that are appropriate for QACs. A detailed presentation regarding the

application of these DDEFs to the determination of Derived No-effect Levels (DNEL) or Reference Doses/Reference Concentrations (RfD/RfC) is beyond the scope of this study. However, a DNEL or RfD/RfC may be derived by dividing the PoD value (e.g., No Observed Adverse Effect Concentration (NOAEC) from a hazard identification study) by the total DDEF. A recent publication illustrates the use of these values to characterize risk to QACs via dermal, inhalation, and oral routes of exposure (Osimitz and Droegge 2021).

Conclusions

The overall database employed to develop our AOP for ADBAC and DDAC as representative QACs is strong. *In vitro* studies provide evidence on the molecular initiating events, where many *in vivo* animal studies are available to characterize the key events leading to AO. When inhaled or ingested, the QACs are incorporated into the epithelial cell membrane at the point of contact. With sufficient dose, the epithelial membrane is disrupted, reducing its fluidity, and releasing cell contents. Further, QACs might disrupt mitochondria of epithelia leading to reduced ATP production. Both events may lead to cell death, either from direct lysis, necrosis, or apoptosis. Pro-inflammatory mediators are recruited to the tissue, resulting in inflammation, edema and enhanced mucus production. The primary tissue-level AO is epithelial degeneration and dysplasia. Most important, no metabolism or distribution is involved in QAC action.

The application of such chemical-specific data for chemicals expressing local, point-of-contact effects has been extensively considered by regulatory agencies worldwide (EPA 2017a, 2017b, 2017c, 2017d; NRC 2001; NRC. 2010a; NRC 2010b, 2016; WHO 2005). In the context of these assessments,

Table 1. DDEFs for QACs.

Uncertainty Factor	Inhalation Route	Oral Route
Inter-species variability		
Toxicokinetics	1	1
Toxicodynamics	1	1 to 3*
Inter-individual (intra-species) variability		
Toxicokinetics	1	1
Toxicodynamics	3	3
Intermediate-to-long term exposures	1	1
OVERALL UF	3	3 to 10

* A value of 3 may be most appropriate given the limited data available on the alteration of microflora.

a set of DDEFs is proposed to replace default UFs for risk assessments on QACs. Such DDEFs not only use the knowledge gained from the many studies available, but are also protective of public health, while enabling continued use of these important beneficial antimicrobials.

Notes

1. For conciseness these chemicals are referred to as ADBAC and DDAC, but, as mentioned above, each of these are groups of chemicals which have similar, but not identical, structures.
2. Heme oxygenase-1, also referred to as HO-1 or heat shock protein 90, is implicated in cytotoxic events and the inflammatory signaling response in alveolar epithelial cells (Rock and Kono 2008; Singal 2010).
3. Rodents do not have an emetic reflex, so emesis would not be expected in these species.

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Data Availability Statement

The authors state that there is no data set associated with the paper. Data sharing is not applicable to this article as no new data were created or analyzed in this study.

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