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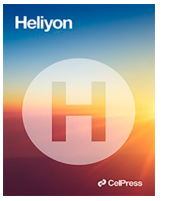

Research article

Adverse outcome pathway-based assessment of pulmonary toxicity from the *in vivo* mixture of biocides dinotefuran and cetylpyridinium chloride

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A R T I C L E I N F O

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A B S T R A C T

Despite the increasing use of biocides globally and their widespread application in various formulations, the understanding of the toxicity of biocide mixtures remains limited. We previously identified cetylpyridinium chloride and dinotefuran as a potential binary biocidal combination associated with pulmonary fibrosis, based on two intersecting adverse outcome pathways (AOPs) using the molecular initiating events (MIE) modeling method and in vitro testing. These compounds activate or inhibit toll-like receptor 4 (TLR4) and peroxisome proliferator-activated receptor-gamma (PPAR-γ), which are associated with pathways having the potential to cause pulmonary fibrosis. In this study, we aimed to validate these AOPs by assessing the toxicity of cetylpyridinium chloride and dinotefuran mixture. Sixty C57BL/6 male mice were exposed to either dinotefuran or cetylpyridinium chloride or a mixture of the two via intratracheal instillation (ITI) to examine the synergistic effects of MIE and key events (KEs) within putative AOPs. Various parameters, including clinical and histopathological indicators, changes in body weight and organ weight, inflammatory cell distribution, and inflammatory cytokine expression in the bronchoalveolar lavage fluid (BALF), were analyzed. Additionally, key indicators such as TLR4, NF-κB, TNF-α were investigated to validate the mechanistic aspects of putative AOPs associated with pulmonary fibrosis. We observed significant changes in body weight and neutrophil count, recognized indicators of inflammation, along with inflammatory cell infiltrates, in the group exposed to the mixture of the two biocides. Moreover, increased levels of markers associated with epithelial–mesenchymal transition (EMT) and fibrosis (TNF-α, Acta2, IL-1β, and MMP9), as well as elevated levels of TGF-β, a common downstream signaling factor of TLR4 and PPAR-γ, were identified. Collectively, our findings highlight the potential toxic effects of a mixture of these two biocides in an in vivo model and confirmed the effective function of the putative AOP.

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1. Introduction

The utilization of biocides across various applications, including insecticides, disinfectants, and preservatives, is steadily expanding globally [1]. Forecasts indicate substantial growth in the biocide market, with Precedence Research [2] predicting a noteworthy compound annual growth rate (CAGR) of 4.70 % from 2023 to 2032, resulting in a market size of approximately 15.35 billion by 2032. The increasing use of biocides reflects a prevailing trend in contemporary society, underscoring the heightened demand for improved public health. Consequently, the global regulation of biocides is becoming progressively more stringent as emerging scientific data continues to inform policy development, thereby enhancing efforts to protect human health and preserve environmental integrity. Additionally, it is crucial to acknowledge that biocides are utilized in mixtures, exposing individual to combination of two or more biocides, thereby emphasizing the imperative need to comprehend the toxicity of such mixtures.

Biocides, such as disinfectants, antiseptics, and preservatives, play crucial roles in various industries and environmental health. They are essential components of antimicrobial formulations and disinfection strategies and contribute significantly to the maintenance of human health and standards of hygiene [3,4]. These substances are widely used in spray-and trigger-type formulations, which often result in dermal and respiratory exposure to aerosolized biocides ([5]; [6]; [7]). Although biocides are vital for controlling harmful microorganisms, their improper use can adversely affect human health. Human exposure to biocides occurs through various routes, including ingestion, dermal contact, and inhalation. Among these, inhalation and skin contact are considered the primary exposure routes [8]. Human exposure to biocides through inhalation, whether from the spraying of products or their evaporation, is considered particularly hazardous [7,9]. Particularly, inhalation, the primary exposure route, contributes to an increased burden of respiratory diseases such as pulmonary fibrosis [10]. Aerosol exposure to biocides in humidifier disinfectants, including polyhexamethylene guanidine, has been identified to induce severe pulmonary fibrosis [11,12]. Therefore, assessing the inhalation toxicity of biocides is essential for identifying potential health hazards.

Biocides are toxic to a wide range of living organisms at relatively high concentrations and cause direct or indirect systemic toxicity, allergenicity, and bacterial resistance [13,14]. Additionally, notable correlations have been observed between the toxicological mechanisms of biocides and their structural properties ([5]; [6]; [15]). Biocidal products comprise a combination of one or more active substances and various formulation additives [16,17]. However, potential adverse effects of biocides, particularly when used in combination with inhalation, remain unclear.

The adverse outcome pathway (AOP) framework has emerged as a valuable instrument for comprehending the intricacies of mixture toxicity. AOPs offer a structured representation of biological events from molecular initiating events (MIE) to adverse outcomes (AO), facilitating an understanding of toxicity mechanisms. Integrating AOP with mixture toxicity presents a potentially comprehensive approach to elucidate the complexities of combined exposures and explore their interactions at the molecular level across diverse chemical groups [18].

To gain further insights, we aimed to elucidate the pulmonary toxicity associated with biocides linked to pulmonary fibrosis. Modeling the effects of chemical mixtures using AOPs helps map the molecular, cellular, and tissue-level events that lead to adverse outcomes like pulmonary fibrosis. By applying AOPs, we can better understand how combined exposures to chemicals, such as cetylpyridinium chloride and dinotefuran, contribute to disease development. This approach is particularly useful for identifying key biological events and pathways affected by mixtures, enabling more accurate predictions of toxicity. Therefore, in this study, we aimed to validate putative AOPs associated with the toxicity of a mixture of dinotefuran and cetylpyridinium chloride, focusing on their correlation with pulmonary fibrosis. Consequently, we previously identified cetylpyridinium chloride and dinotefuran as potential biocides linked to pulmonary fibrosis through an integrated analysis of adverse outcome pathways (AOPs) and quantitative structure–activity relationship (QSAR) modeling [19]. To elucidate potential mixture toxicity, including the synergistic effects of these biocides associated with pulmonary toxicity, 60 male C57BL/6 mice were individually exposed to each biocide and to a combination of the two through intratracheal instillation (ITI). Following intratracheal instillation of both substances, comprehensive analyses, including clinical, histopathological, and molecular assessments, were conducted.

Table 1
Experimental design for the ITI administration of dinotefuran and cetylpyridinium chloride in C57BL/6 mice.

Group	Subgroup	Dose		No. of animals
		Dinotefuran (mg/kg)	Cetylpyridinium chloride (mg/kg)	
Vehicle Control Group	VC-1	0	0	6
	VC-2	0	0	6
Single Treated Group: Dinotefuran	Dino.-1	50	0	6
	Dino.-2	100	0	6
Single Treated Group: Cetylpyridinium chloride	Cetyl.-1	0	0.031	6
	Cetyl.-2	0	0.0625	6
	Cetyl.-3	0	0.125	6
	Cetyl.-4	0	0.250	6
Mixture Treated Group	Mix.-1	100	0.031	6
	Mix.-2	100	0.0625	6

VC-1; vehicle control for dinotefuran, VC-2; vehicle control for cetylpyridinium chloride.

2. Materials and methods

2.1. Animals

For this study, 7-week-old C57BL/6 male mice were purchased from Orient Bio Inc. (Seongnam, Korea). The mice were housed in controlled conditions of temperature (22 ± 3 °C), light (12-h light: 12-h dark cycle), relative humidity (40–60 %), and air ventilation rate (10–20 times/h). The mice were fed sterilized pellet food (PMI Nutrition International, Richmond, VA, USA) and tap water ad libitum. All the animal experimental procedures were approved by the Institutional Animal Care and Use Committee (IACUC) of the Korea Institute of Toxicology (IACUC approval No.: 2009-0287).

2.2. Experimental design

The experimental animals were divided into four groups: 1) vehicle control (VC), 2) dinotefuran (CAS No. 165252-70-0), 3) cetylpyridinium chloride (CAS No. 123-03-5), and 4) dinotefuran and cetylpyridinium chloride mixture-exposed group (Table 1).

The mice were randomized into groups of six individuals using the Pristima System Ver. 6.4 (Xybion Medical Systems Co., USA). Dinotefuran was administered once daily for seven consecutive days, whereas cetylpyridinium chloride was administered once daily on days 1, 4, and 7. In the mixture-exposed group, dinotefuran and cetylpyridinium chloride were administered using the same protocol. Cetylpyridinium chloride was administered 30 min after dinotefuran administration. Saline was used as the vehicle control for both compounds, and fresh solutions were prepared daily to ensure consistency and stability. The administration was performed via intratracheal instillation (ITI) using an automatic video instillator, and animals were anesthetized with 2–5% isoflurane prior to each administration. Mice were euthanized one day (24 h) after the last ITI administration. The experimental scheme is shown in Fig. S.1. Dinotefuran was purchased from TCI (Tokyo, Japan) and cetylpyridinium chloride was purchased from Sigma-Aldrich (St. Louis, MO, USA).

2.3. Analysis of bronchoalveolar lavage fluid (BALF)

Twenty-four hours after the last biocide instillation, the mice were euthanized with an overdose of isoflurane with continued exposure for 1 min once breathing stopped. BALF was collected thrice from the right lung using 0.7 mL of calcium- and magnesium-free PBS (pH 7.4) in a tracheal tube. The total number of cells in the collected BALF was counted using a NucleoCounter (NC-250, ChemoMetec, Gydevang, Denmark). For differential cell counts, BALF cells were prepared using Cytospin (Thermo Fisher Scientific, Inc., Waltham, MA, USA) and stained with Diff-Quik solution (Dade Diagnostics, Aguada, Puerto Rico). BALF was immediately centrifuged at 2,000 rpm for 5 min, and the collected supernatant was stored at −70 °C.

2.4. Quantitative reverse transcription polymerase chain reaction (qRT-PCR)

Total RNA was extracted from lung tissues using TRIzol reagent (Invitrogen, USA), following the manufacturer's instructions. Total RNA was quantified using a NanoDrop 2000 spectrophotometer (Thermo Fisher Scientific, Wilmington, DE, USA). After RNA extraction, the quality check results (260/230 ratios) were both 2.0 or higher, indicating high RNA purity. The relative mRNA expression levels were quantified using a QuantStudio5 Real-Time PCR system with the SYBR Green PCR Master Mix (Applied Biosystems, Foster City, CA, USA). The cycling conditions were as follows: 95 °C for 10 min, followed by 40 cycles of 95 °C for 15 s, and 59 °C for 1 min. The cycle threshold method was used to calculate the relative changes in target gene expression using the QuantStudio Design & Analysis v. 1.4 software (Applied Biosystems, Foster City, CA, USA). The mRNA levels of each gene were normalized to those of Gapdh. The primer sequences used for qRT-PCR are listed in Table S.1.

2.5. Immunohistochemistry (IHC)

Immunohistochemical analyses were performed using the R.T.U. Vectastain Universal Quick Kit instructions (Vector Laboratories, Burlingame, Calif, USA) according to manufacturer's instructions. Briefly, the slides were incubated with Endo/Blocker (Biomeda Corp, Foster City, Calif) for 15 min and Proteinase K (Dako, Glostrup, Denmark) for 15 min at 37 °C. The slides were then incubated with normal horse serum for 30 min at room temperature. The slides were probed with anti-TLR4(Novus Biologicals, Littleton, CO, USA), anti -PPAR-gamma (Abcam, Cambridge, MA, USA) antibodies for 2 h at room temperature, followed by incubation with pre-diluted biotinylated pan-specific IgG for 30 min. The slides were incubated in streptavidin/peroxidase complex reagent for 15 min and then in a 3-amino-9-ethylcarbazole substrate kit for 5 min. Lung tissue sections from mice that were incubated in the absence of the primary antibody were used as controls. The slides were analyzed after immunostaining using an Axio Imager M1 (Carl Zeiss, Oberkochen, Germany). As previously described (Wilson et al., 2010), the degree of immunoreactivity was scored on a scale of 0–4.

2.6. Histopathological analysis

To examine the histopathological alterations, the lung tissues of mice were fixed in 10 % (v/v) neutral buffered formalin. The samples were then dehydrated, embedded in paraffin, and sectioned at a thickness of 4 μm, deparaffinized in xylene, and stained with hematoxylin and eosin (H&E), or Masson's Trichrome (MT) stain (Sigma-Aldrich, USA). Stained sections were analyzed under a light

microscope (Axio Imager M1; Carl Zeiss, Oberkochen, Germany). Each consecutive field was individually scored for the severity of inflammatory cell infiltration, perivascular/bronchiole eosinophilic cell infiltration, mucus production, goblet cell hyperplasia, and fibroplasia. The degree of lung injury was determined using a semi-quantitative score (0–4). Histological grading was performed by an experienced histopathologist using a blinded scoring system to determine the extent and severity of inflammation and goblet cell hyperplasia as previously described (Shackelford et al., 2002).

2.7. Statistical analyses

Statistical analyses were performed using GraphPad Prism version 7 (GraphPad Software, San Diego, CA, USA). All data are represented as the mean ± standard deviation (SD). The data were tested for normality with the Shapiro-Wilk test. Statistical comparisons were performed using one-way analysis of variance (ANOVA), followed by Dunnett- and Bonferroni-adjusted Mann–Whitney U tests. Statistical significance was set at $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$, $^{****}P < 0.0001$.

3. Results

3.1. Changes in body weight and lung weight of mice exposed for 7 days to dinotefuran and cetylpyridinium chloride, either alone or in combination

We observed the mortality and alterations in body and lung weights during the exposure period to evaluate the induction of lung injury. No mortality was observed in any dose group, including the combination-exposure group treated with Dino. and Cetyl. A significant decrease in body weight was observed in the combination-exposure group (Dino. + Cetyl. 0.062 mg/kg) (Fig. 1A, Table S.2.).

Relative lung weight changes were significantly increased in the Cetyl. 0.25 mg/kg group compared with that in the control group. However, no significant increase in lung weight was observed with combined exposure in contrast to the single exposure of either Dino. or Cetyl. (Fig. 1B).

3.2. Total and differential cell analysis of bronchoalveolar lavage fluid from mice exposed for 7 days to dinotefuran (Dino) and cetylpyridinium chloride (Cetyl), either alone or in combination

To determine the biocide exposure-induced pulmonary inflammatory responses, we analyzed the total and immune cell composition in the BALF. No significant changes in total cell counts were observed in the all Dino. exposure groups compared with that in the control group; however, a significant increase was observed in the 0.125 and 0.25 mg/kg Cetyl. groups compared with that in the control group (Fig. 2A). Furthermore, a significant increase in the total cell count was observed in the Dino. and Cetyl. Combination-exposure group (100 mg/kg of Dino. and 0.062 mg/kg of Cetyl.) compared with that in the control group; however, no significant changes were observed in contrast to that in the individual exposure group.

The immune cell composition of BALF revealed that macrophages were the predominant cell type in the control group but were significantly decreased in the 0.25 mg/kg Cetyl. group. Additionally, a significantly increased proportion of lymphocytes in the 0.25 mg/kg Cetyl. exposure group compared to the control group. Moreover, we also observed a significant increase in the number of neutrophils in the combination-exposure group (100 mg/kg of Dino. and 0.031 mg/kg of Cetyl.) compared with that in the single-exposure group, suggesting a synergistic inflammatory response induced by combined exposure of the two biocides (Fig. 2B).

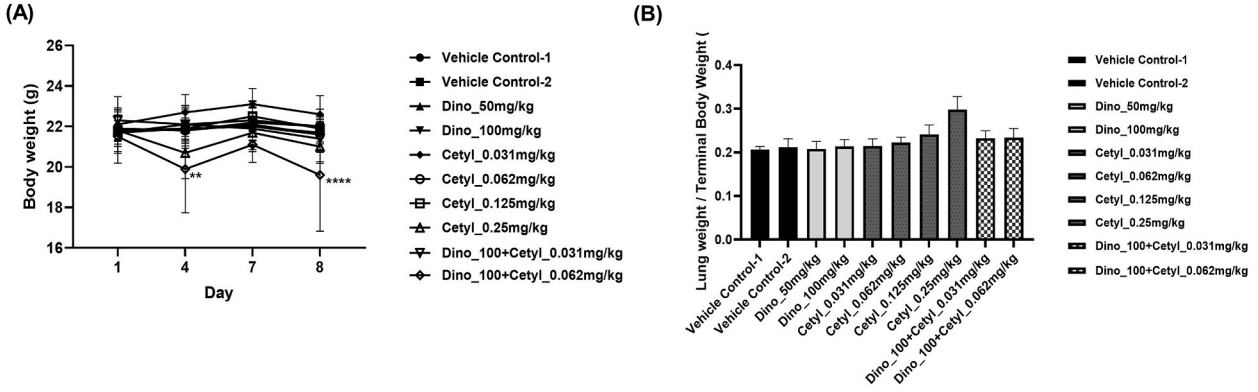

Fig. 1. Effect of a 7-day exposure to dinotefuran (Dino) and cetylpyridinium chloride (Cetyl), either alone or in combination, on body weight and lung weight in mice. Changes in (A) body weight and (B) relative lung weight in Dino.- and Cetyl.-exposed mice. Relative lung weight (%) was calculated as the ratio of lung weight to total body weight. Data are presented as mean ± SD (n = 6/group). $^{**}P < 0.01$, $^{****}P < 0.0001$ vs. vehicle control.

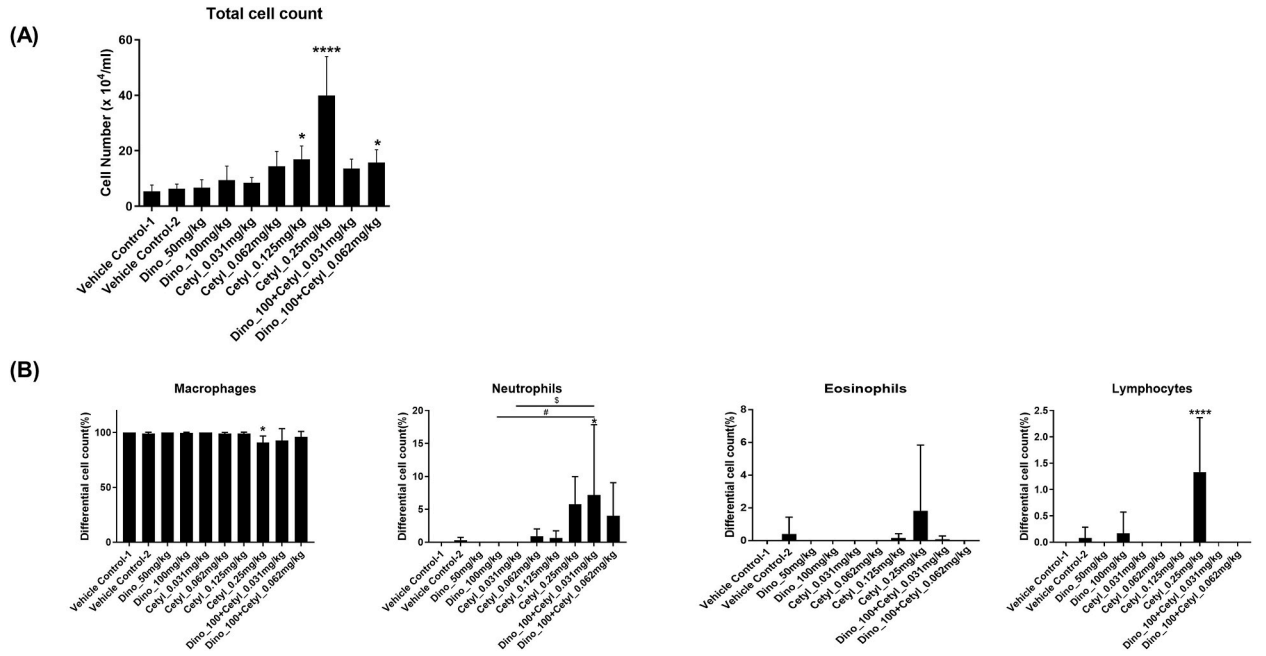

Fig. 2. Bronchoalveolar lavage fluid (BALF) analysis after a 7-day exposure of mice to dinotefuran (Dino) and cetylpyridinium chloride (Cetyl), either alone or in combination. (A) Total and (B) differential cell counts in BALF of Dino.- and Cetyl-exposed mice. The data are presented as mean ± SD (n = 6/group). *$P < 0.05$, ****$P < 0.0001$ vs. vehicle control; $^{\#}P < 0.05$ vs. Dino. group; $^{\$}P < 0.05$ vs. Cetyl. group.

3.3. Pulmonary histopathological changes in mice exposed for 7 days to dinotefuran (Dino) and cetylpyridinium chloride (Cetyl), either alone or in combination

No significant histopathological changes were observed in mice in the all Dino. exposure group compared with that in the control group. However, the 0.25 mg/kg Cetyl. exposure group exhibited significant histopathological changes, including inflammatory cell infiltration, granulomatous inflammation, and pulmonary fibrosis. Furthermore, notable changes in inflammatory cell infiltration were observed in the combination-exposure group compared with that in single-exposure groups (Fig. 3). These results suggest a synergistic effect of this combination on the inflammatory response.

3.4. Identification of MIE involved in the putative AOP induced by a 7-day exposure of mice to dinotefuran (Dino) and cetylpyridinium chloride (Cetyl), either alone or in combination

To validate the MIE of the putative AOP mechanism, we performed immunohistochemical analyses of targets associated with pulmonary fibrosis, such as toll-like receptor 4 (TLR4) and peroxisome proliferator-activated receptor-gamma (PPAR-γ), following exposure to Dino. and Cetyl. A significant increase in the expression of TLR4 was observed in the combination-exposure group compared with that in single-exposure groups (Fig. 4A). We observed significantly increased levels of PPAR-γ in Cetyl. exposure group compared with that in the control group. However, no synergistic effects were observed in the combination-exposure group compared with that in the single-exposure group (Fig. 4B).

3.5. Analysis of molecular markers associated with KEs and AOs in the putative AOP mechanism following a 7-day exposure of mice to dinotefuran (Dino) and cetylpyridinium chloride (Cetyl), either alone or in combination

Using qRT-PCR, we examined the expression of KEs associated with a putative AOP, such as TGF-β, NF-κB, and ACTA2, in the lung tissues following exposure to dinotefuran and cetylpyridinium chloride, either alone or in combination. The results of the melting curve analysis are provided in the supplementary data to confirm the specificity of the PCR products. Notably, we observed no significant changes in TGF-β, corresponding to downstream signals of MIEs, TLR4 and PPAR-γ, in the Dino. exposure group. However, Tgfb mRNA expression significantly increased in the Cetyl. exposure group compared with that in the control group. Additionally, the Nfkb mRNA expression was significantly elevated in the combination-exposure group compared with that in the control group. Acta2 expression was significantly increased following Cetyl. exposure. Furthermore, a significant increase in Tgfb, Nfkb, and Acta2 mRNA expression was observed in the combination-exposure group, suggesting a synergistic effect (Fig. 5A).

Subsequently, we investigated the markers of putative AOs (TNF-α, IL-1β, and MMP9) associated with lung fibrosis induced by Dino. and Cetyl. exposure. Tnfa mRNA expression significantly increased in the Dino. and Cetyl. exposure group compared with that in the control group. No significant change in IL-1β was observed in Dino. and Cetyl. exposure group. Moreover, MMP9, which plays a key

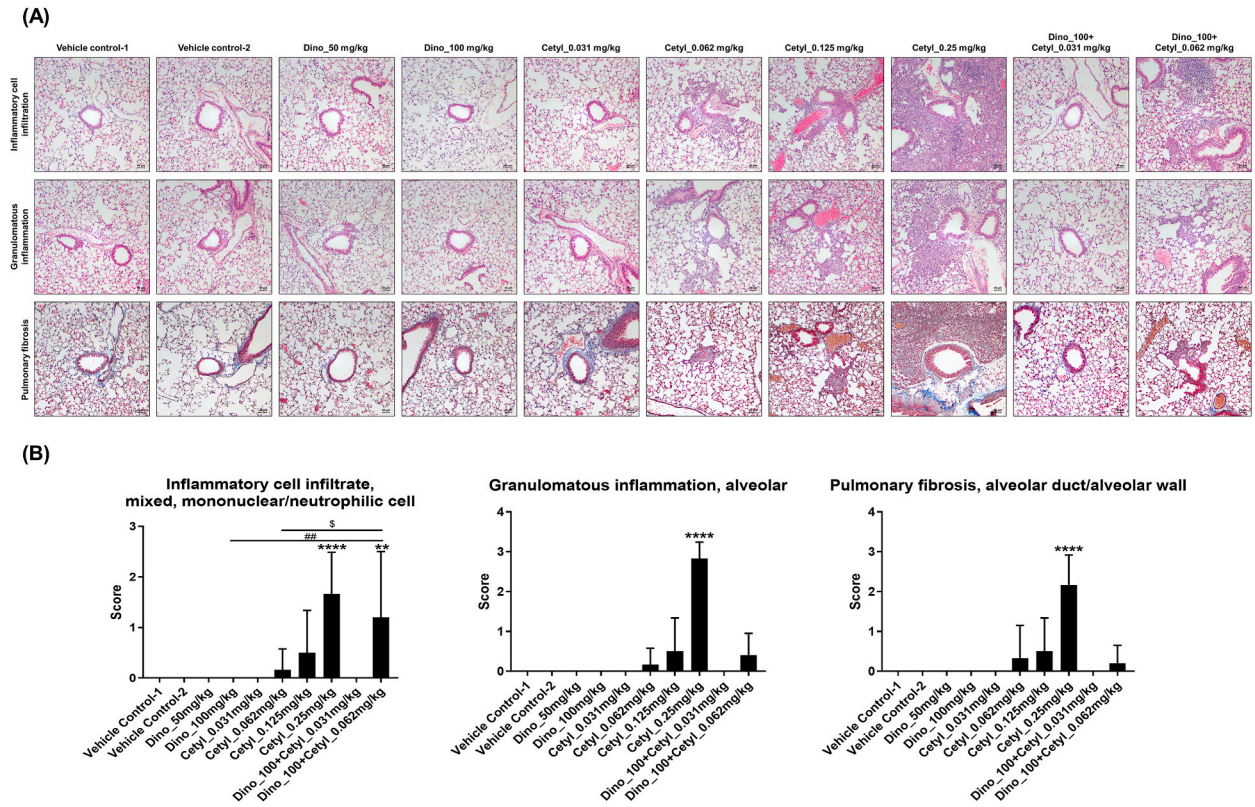

Fig. 3. Histopathological changes in the lungs of mice after a 7-day exposure to dinotefuran (Dino) and cetylpyridinium chloride (Cetyl), either alone or in combination. The score is the average of grading scores per group. Hematoxylin and eosin (H&E) staining at magnification × 200. Pulmonary fibrosis was evaluated using MT staining at × 200 magnification. $^{**}P < 0.01$, $^{****}P < 0.0001$ vs. Vehicle control; $^{\#\#}P < 0.01$ vs. Dino. group; $^{\$}P < 0.05$ vs. Cetyl. group.

role in the progression of lung fibrosis, was not significantly altered in the Dino. exposure group. However, expression was significantly increased in the Cetyl. exposure group compared with that in the control group. Furthermore, there was a significant increase in TNF-α, IL-1β, and MMP9 in response to Dino. and Cetyl. combination exposure (Fig. 5B). These results suggest that the combination of Dino. and Cetyl. induces a synergistic effect on the molecular markers of putative AOP at the transcriptional level.

4. Discussion

This study focused on two significant compounds: Dino. and Cetyl. The current study builds on a previous work that identified the combination of dinotefuran and cetylpyridinium chloride as a potential inducer of pulmonary fibrosis. Both are known biocides that modulate the TLR4 and PPAR-γ pathways associated with pulmonary fibrosis. Based on an integrated analysis of AOPs), QSAR modeling for MIEs, and in vitro assays, this study aimed to validate putative AOPs by evaluating the toxicity of a mixture of dinotefuran and cetylpyridinium chloride associated with pulmonary fibrosis.

Preliminary testing was conducted to establish the administration conditions of Dino. and Cetyl. Based on the pre-test results, Dino. was administered once daily for a total of three administrations into the respiratory tract. No significant toxic effects were observed at the highest dose of Dino. (100 mg/kg) compared to the control group (data not shown). In contrast, toxicological effects were observed in the Cetyl. group following three administrations. Therefore, Dino. was subsequently administered once a day for a total of seven times, and Cetyl. were administered once a day for a total of three times. For Cetyl., the doses used in this study are very similar to real-world human intake of cetylpyridinium chloride. According to European Union Scientific Committee on Consumer Safety (SCCS), the total absorption of cetylpyridinium chloride by various routes in adults is about 0.114 mg/kg body weight/day in 2017[20]. In case of Dino., the literature and reports were reviewed and the highest dose that could be formulated in saline, 100 mg/kg, was selected as there were no reports of human exposure level. This dose is similar to the LOAEL of 60 mg/kg/day obtained in a previous 28-day inhalation toxicity study in rats [21].

Inhalation exposure is a natural route, as in human exposure through most sprayable biocide products, but this technique cannot always be used because inhalation equipment and a large quantity of test substance are needed. In addition, co-exposure through oral and skin routes cannot be disregarded. As a result of these restrictions, intratracheal instillation has been widely used. Intratracheal instillation is an alternative method for inhalation and is possible to ensure a uniform dose exposure. The method in intratracheal

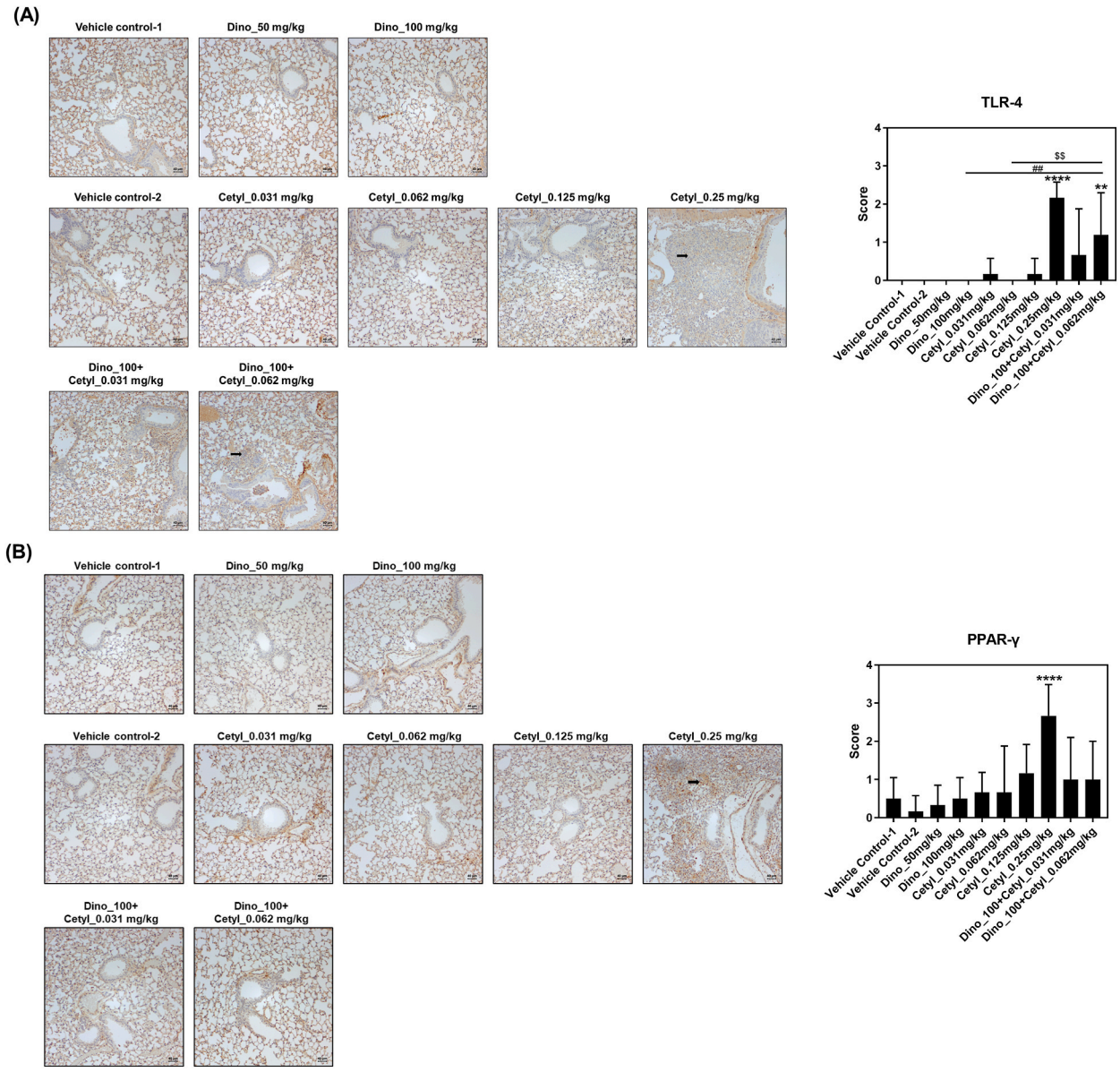

Fig. 4. Immunohistochemical (IHC) analysis of (A) TLR4 and (B) PPAR-γ expression in lung tissues of mice exposed for 7 days to dinotefuran (Dino) and cetylpyridinium chloride (Cetyl), either alone or in combination. $^{**}P < 0.01$, and $^{****}P < 0.0001$ vs. vehicle control; $^{\#\#}P < 0.01$ vs. Dino. group; $^{\$\$}P < 0.01$ vs. Cetyl. group. IHC staining at magnification × 200. The scale bar indicates a measurement of 40 μm in all images. The black arrow indicates the positive cells between the target (TLR4, PPAR-γ) antigens and their antibody.

instillation studies is to expose the chemical substance directly through the trachea, and they are suitable for not only the elucidation of dose-response relations with a known quantity dosage but also for the elucidation of lung disorders induced by chemical substances [22]. Since the purpose of the current study is to identify and elucidate the potential mixture toxicity, including the synergistic effects of biocides associated with pulmonary toxicity, the intratracheal instillation method is sufficiently reasonable.

This study was designed to observe the qualitative and quantitative synergistic effects of the MIE and KE on the putative adverse outcome (AO) during combined exposure to two biocides by evaluating parameters such as mortality, body and lung weight, histopathological findings, and distribution of inflammatory cells in bronchial lavage fluid.

We observed significant changes in body weight, increased neutrophil levels (an indicator of inflammation), and prominent inflammatory cell infiltration in lung tissue were observed in the group exposed to the biocide mixture. Elevated levels of markers associated with epithelial–mesenchymal transition and fibrosis, such as TNF-α, Acta2, IL-1β, and MMP9, were identified, along with increased levels of TGF-β, a pivotal downstream signaling factor of both TLR4 and PPAR-γ (Fig. 6 and Table S.3.). No significant changes in fibrosis-related histological findings were observed following combined exposure under the current experimental

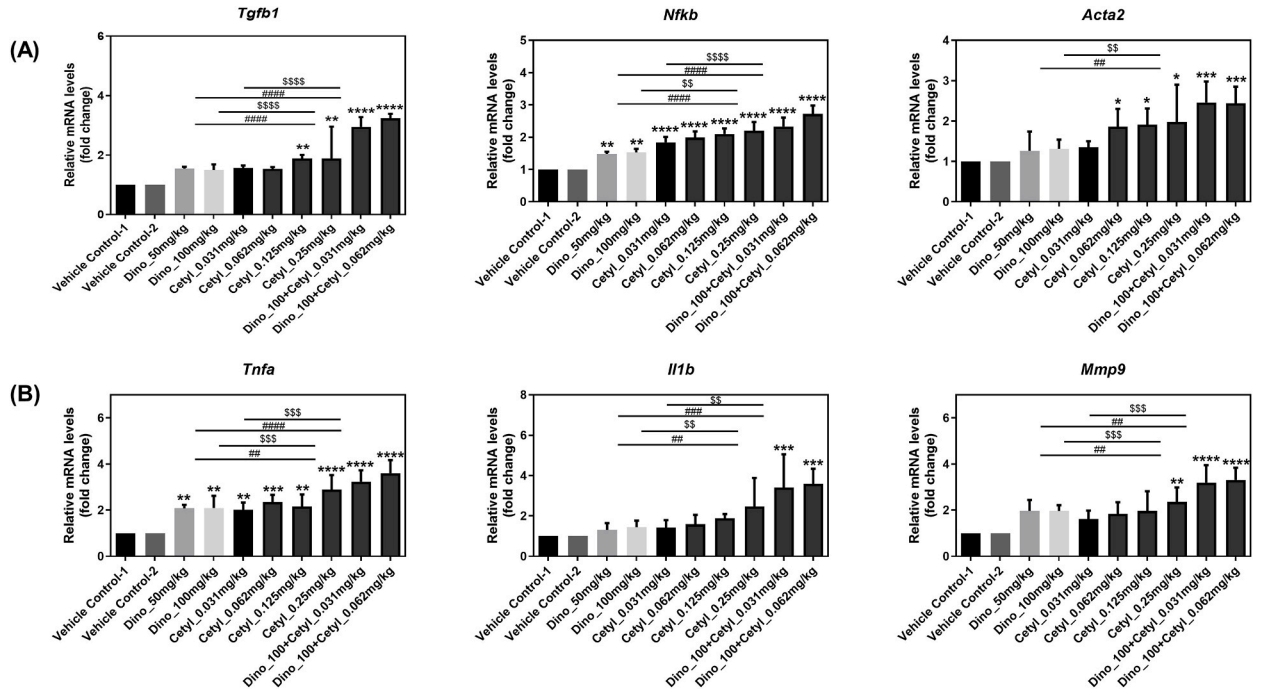

Fig. 5. Relative mRNA levels of targets of (A) key events (KE; *Tgfb, Nfkb, and Acta2*) and (B) adverse outcomes (AO; *Tnf-α, Il-1β, and Mmp9*) in lung tissue of mice exposed for 7 days to dinotefuran (Dino) and cetylpyridinium chloride (Cetyl), either alone or in combination. * indicates the comparison with vehicle control, # denotes the comparison with Dino. exposure group, and $ designates the comparison with Cetyl. exposure group. The numerical annotations are as follows: one indicates the statistical significance at $P < 0.05$, two indicates $P < 0.01$, and four indicates $P < 0.0001$.

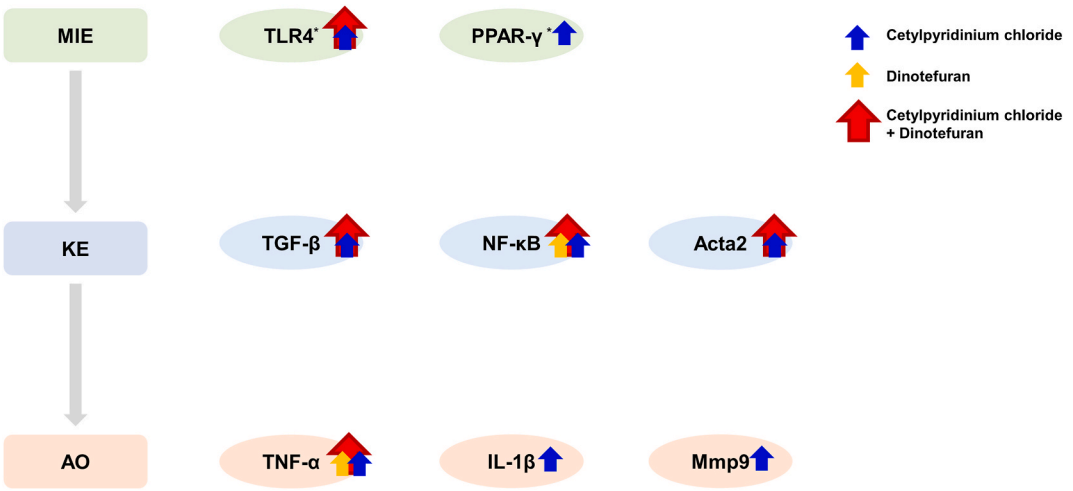

Fig. 6. Summary of the assessment of concordance between events (MIE, KE, and AO) in the putative AOP analysis. * indicates significant changes in protein expressions, while all other markers represent changes in mRNA expression levels.

conditions. However, molecular-level alterations such as inflammatory cell infiltration indicate the potential for pulmonary toxicity induced by the combination of Dino and Cetyl. While these findings highlight possible synergistic effects at the molecular level, it is important to note that no conclusive evidence of pulmonary fibrosis was observed in this short-term exposure model. Therefore, the results suggest that combined exposure to Dino and Cetyl could induce pulmonary toxicity, but further studies with prolonged exposure and detailed mechanistic investigations are necessary to evaluate the potential for fibrosis development over time.

In this study, we acknowledge the important concern that mRNA levels do not always directly correspond to protein levels. This assumption, often made in gene expression studies, overlooks the complex layers of regulation that occur post-transcriptionally,

including mRNA splicing, stability, degradation, and translation efficiency [23]. Consequently, while mRNA measurements provide valuable insights into gene regulation and potential protein synthesis, they do not necessarily reflect the functional output at the protein level. Given this limitation, the results of this study must be interpreted with caution. While changes in mRNA levels can indicate alterations in gene expression, further validation at the protein level is essential to fully understand the biological consequences. Future studies should incorporate the analyses to correlate mRNA and protein expression directly, as this would provide a more comprehensive understanding of the functional impacts of gene regulation.

In conclusion, while mRNA data offer a valuable snapshot of transcriptional activity, they must be complemented by protein-level measurements to accurately assess the functional outcomes of gene expression changes.

The current study has several limitations. First, intratracheal instillation is not a satisfactory alternative to inhalation and may result in markedly different distribution, transport, and toxicity of the test substances in the lungs. However, our previous study revealed that intratracheally instilled animals were reported to exhibit histological features similar to that of inhaled animals [24]. Therefore, we propose that intratracheal instillation is an acceptable model for observing the synergistic effects of biocides associated with pulmonary toxicity, however, the associated results require further validation using inhalation studies. Second, studies are needed that reflect real-world scenarios (concentration and frequency of exposures). Third, in this study, each test substance was administered separately, as it was deemed that mixing the two test substances might alter their physicochemical properties. However, in actual product use scenarios, exposure is more likely to occur in the form of mixtures, and it is therefore necessary to assess the toxicological effects of mixture types of substance. Fourth, although this study identified synergistic effects of two chemicals based on AOPs on lung fibrosis, animal models may not reproduce all of the complex characteristics of human lung fibrosis, and direct molecular comparisons between human disease and animal models are limited. Further research is required that reflect real-world exposure scenarios to dinotefuran and cetylpyridinium chloride using models that better mimic human lung fibrosis characteristics.

Overall, we elucidated the toxicity of a mixture of dinotefuran and cetylpyridinium chloride, providing valuable insights into the molecular mechanisms and synergistic effects that lead to pulmonary fibrosis. Beyond focusing on dinotefuran and cetylpyridinium chloride, this study contributes to the understanding of chemical risk assessment based on a systemic approach to AOPs. Additionally, these results suggest that integrating the verified AOPs into the biocidal material development stage provides a robust framework for efficient toxicological screening, offering a deeper understanding of the potential toxicity and disease outcomes associated with combination exposure. Further research is required to elucidate the molecular mechanisms underlying the pulmonary toxicity of dinotefuran and cetylpyridinium chloride, particularly in combination exposures.

CRediT authorship contribution statement

Yoon Cho: Writing – review & editing, Writing – original draft, Investigation. **Mi-Kyung Song:** Investigation, Data curation, Conceptualization. **Dong Im Kim:** Investigation, Formal analysis, Data curation. **Min-Seok Kim:** Resources, Project administration, Investigation, Conceptualization. **Kyuhong Lee:** Supervision, Project administration, Funding acquisition.

Ethics approval statement

The animal experiment approved by the Institutional Animal Care and Use Committee (IACUC) of the Korea Institute of Toxicology (Approval No.: 2009-0287).

Data availability statement

Data are available within the article. The data associated with this study have not been deposited into a publicly available repository, it will be made available upon request.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.heliyon.2025.e42134.

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