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Inflammatory effects induced by selected limonene oxidation products: 4-OPA, IPOH, 4-AMCH in human bronchial (16HBE14o-) and alveolar (A549) epithelial cell lines

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HIGHLIGHTS

- Cytotoxicity and induction of inflammation by selected limonene oxidation products were assessed in human lung cell lines.
- 4-OPA demonstrated the strongest cell viability destruction of A549 and 16HBE14o- cells.
- IPOH was stimulating IL-6, IL-8 and TNF-α release from both A549 and 16HBE14o- cell lines.
- 4-OPA and 4-AMCH were inhibiting IL-6, IL-8, TNF-α release in 16HBE14o-cells, but not in A549 cells.

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ABSTRACT

Limonene, a monoterpene abundantly present in most of the consumer products (due to its pleasant citrus smell), easily undergoes ozonolysis leading to several limonene oxidation products (LOPs) such as 4-acetyl-1-methylcyclohexene (4-AMCH), 4-oxopentanal (4-OPA) and 3-isopropenyl-6-oxoheptanal (IPOH).

Toxicological studies have indicated that human exposure to limonene and ozone can cause adverse airway effects. However, little attention has been paid to the potential health impact of specific LOPs, in particular of IPOH, 4-OPA and 4-AMCH.

This study evaluates the cytotoxic effects of the selected LOPs on human bronchial epithelial (16HBE14o-) and alveolar epithelial (A549) cell lines by generating concentration-response curves using the neutral red uptake assay and analyzing the inflammatory response with a series of cytokines/chemokines.

The cellular viability was mostly reduced by 4-OPA [IC_{50} = 1.6 mM (A549) and 1.45 mM (16HBE14o-)] when compared to IPOH [IC_{50} = 3.5 mM (A549) and 3.4 mM (16HBE14o-)] and 4-AMCH [IC_{50} could not be calculated]. As a result from the inflammatory response, IPOH [50 μM] induced an increase of both IL-6 and IL-8 secretion in A549 (1.5-fold change) and in 16HBE14o- (2.8- and 7-fold change respectively). 4-OPA [50 μM] treatment of A549 increased IL-6 (1.4-times) and IL-8 (1.3-times) levels, while in 16HBE14o- had an opposite effect. A549 treated with 4-AMCH [50 μM] elevate both IL-6 and IL-8 levels by 1.2-times, while in 16HBE14o- had an opposite effect.

Based on our results, lung cellular injury characterized by inflammatory cytokine release was observed for both cell lines treated with the selected chemicals at concentrations that did not affect their cellular viability.

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1. Introduction

The prevalence of respiratory diseases, asthma and allergies in large populations has increased substantially in recent decades (Tarlo and Lemiere, 2014; Melén et al., 2008). This phenomenon has been associated, among other factors, to the several chemicals used in various commercially available consumer products (*e.g.* cleaning agents, air-fresheners) that are released into the everyday environment (Crinnion, 2012; Heinrich, 2011; Patelarou et al., 2015; Choi et al., 2010; U.S. Food and Drug Administration, 2009).

Limonene, a common terpene added to a variety of consumer products intended for occupational and personal use, is a very reactive compound towards ozone (O_3), hydroxyl (OH) and peroxy (RO_2) radicals. Its reaction with ozone leads to various limonene oxidation products (LOPs) such as 4-acetyl-1(+/−) methylcyclohexene (4-AMCH), 4-oxopentanal (4-OPA) and 3-isopropenyl-6-oxoheptanal (IPOH) (Hakola et al., 1994; Wells, 2012; Forester and Wells, 2009). Physico-chemical properties of the selected LOPs are summarized in Table 1.

The presence of 4-OPA has been detected in the forestal atmosphere ranging from 180 to 1570 ng m^{-3} (44–384 pptv) (Matsunaga et al., 2004), in a simulated indoor air office at levels around of 2.3 ppb (ozone concentration was 16 ppb) (Wisthaler and Weschler, 2010) and in a simulated aircraft cabin up to 7 ppb (ozone concentration was 61–77 ppb) (Weschler et al., 2007). Nørgaard et al. (2014) measured the concentrations of 4-OPA, IPOH and 4-AMCH in five european offices using different floor cleaning agents (containing high and low total volatile organic compounds (VOCs)). Before the replacement of a regular floor cleaning agent (high total VOCs) and independently of the countries where measurements were performed, concentrations of 4-OPA, IPOH, and 4-AMCH up to 18 μg m^{-3}, 10 μg m^{-3} and 0.1 μg m^{-3}, respectively were found (Nørgaard et al., 2014).

Although several studies have indicated that humans or animals exposure to terpene ozonolysis reaction products is associated with respiratory diseases (*e.g.* asthma) (Sundell et al., 2004) and cardiovascular disease (Uzoigwe et al., 2013; Pope et al., 2004), only a few studies (including both *in vivo* and *in vitro* experiments) available in the literature directly address the impact of selected LOPs on human health.

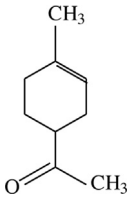
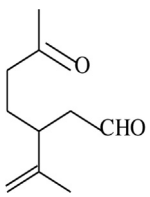
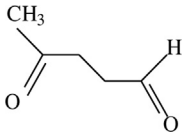
An *in vivo* study has been carried out in a head out mouse bioassay where the respiratory effects caused by 4-OPA, IPOH, 4-AMCH were investigated (Wolkoff et al., 2013). Based on the different respiratory parameters such as time of brake (TB), time of inspiration/expiration (TI/TE) and mild expiratory flow rate, the authors reported that IPOH might be classified as a sensory irritant as observed by the elongation of TB, and with an estimated no observed (adverse) effect level (NO(A)EL) of around 1.6 ppmv, while for 4-AMCH, the NOEL for sensory irritation was established to be around 13 ppmv. When mice were exposed to 4-OPA, effects such as airflow limitation, sensory and pulmonary irritation were noticed, with an estimate for sensory irritation of around 3.4 ppmv.

Anderson et al. have examined the inflammatory and allergic responses in mice exposed to 4-OPA through both dermal and pulmonary routes of exposure (Anderson et al., 2012). Using relatively high doses (0.08–1.97 mM for dermal exposure), the authors identified 4-OPA as being irritant (*e.g.* at 1.97 mM 4-OPA, $p < 0.01$) and sensitizer (*e.g.* 0.02 mM, $p < 0.01$). Pulmonary toxicity in mice following exposure to 0.08 mM 4-OPA, enhanced airway responsiveness, neutrophil and lymphocytes influx, and interferon-γ production by lung-associated lymph nodes.

On the other hand, one *in vitro* study assessed the potential inflammatory capacity of 4-OPA by exposing the pulmonary epithelial cells (A549) to a gas phase containing 65 ppm 4-OPA (Anderson et al., 2010). Inflammatory markers levels of IL-8 and TNF-alpha were both found to be elevated after 4-OPA exposure at all time points (8, 12, 24 h), while IL-6 and GM-CSF significantly increased at 12 h (*e.g.* 1059 pg mL^{-1} for IL-6 and 17 pg mL^{-1} for GM-CSF).

The aim of the present study therefore was to investigate the inflammatory effects of 4-OPA, IPOH and 4-AMCH on human pulmonary cell lines: bronchial epithelium (16HBE14o-) and

Table 1
Physico-chemical properties of selected limonene ozonolysis products: 4-acetyl-1-methylcyclohexene (4-AMCH), 3-isopropenyl-6-oxo-heptanal (IPOH), 4-oxopentanal (4-OPA) (ChemSpider web page, 2016).

LOPs	CAS no.	MW	Vapor pressure mmHg	Boiling point °C	Water solubility mg L^{-1}	Structure
4-acetyl-1-methylcyclohexene $C_9H_{14}O$, (4-AMCH)	6090−09-1	138.21	0.568	205–206	811	
3-isopropenyl-6-oxo-heptanal $C_{10}H_{16}O_2$, (IPOH)	7086−79-5	168.23	1.3	80.5–82/ 1 mm Hg	2920	
4-oxopentanal $C_5H_8O_2$, (4-OPA)	626−96-0	100.12	1.3	100–105/ 20 mm Hg	50000	

alveolar epithelium (A549). The target chemicals were selected on the basis of their stability compared to other limonene ozonolysis products such as peroxides, their general abundance with high ozone or hydroxyl radical yields (Forester and Wells, 2009) and on the hypothesis that terpene reaction products containing multiple oxygen groups such as dicarbonyls may cause inflammatory and respiratory sensitizing effects and/or skin irritation (Pope et al., 2004; Wolkoff et al., 2013; Anderson et al., 2012; Anderson et al., 2010; Anderson et al., 2007).

The human lung cell line A549 is commonly used as a Type II pulmonary epithelial cell model, and was thus selected to assess the lung toxicity of the three LOPs. A recent overview of cell-based models for pulmonary research is available (Gordon et al., 2015). The human bronchial epithelial cell line 16HBE14o- was included in these experiments, due to its capacity to react to inflammatory stimuli like the primary human bronchial epithelial cells (BéruBé et al., 2010).

Biological endpoints such as cell viability assessed by neutral red uptake assay (NRU) and production of inflammatory mediators such as IL-8, IL-6, TNF-alpha, IL-10 and IL-13 were determined after 24 h at different concentrations for each chemical. Moreover, gas chromatography-flame ionization detection (GC-FID) was used to check chemicals stability in the culture medium.

2. Materials and methods

2.1. Chemical reagents and materials

Acetonitrile, 4-oxopentanal (4-OPA), 3-isopropenyl-6-oxoheptanal (IPOH) and 4-acetyl-1-methylcyclohexene (4-AMCH), sodium dodecyl sulphate were obtained from Sigma Aldrich (Sigma Aldrich, St. Louis MO, USA).

2.2. Biological reagents and materials

Roswell Park Memorial Institute medium (RPMI 1640), Minimum Essential Medium (MEM), fetal bovine serum (FBS), penicillin-streptomycin, 0.25% trypsin/EDTA, phosphate buffered saline (PBS) and 4-2-hydroxyethyl-1-piperazinyl-ethanesolfonic acid (HEPES) were purchased from Invitrogen (USA). Mammalian protein extraction reagent (M-PER) was purchased from Thermo Fischer (Italy).

The human lung epithelial carcinoma cell line (A549) was obtained from American Type Culture Collection (ATCC #: CCL-185). The human bronchial epithelial cell line (16HBE14o-) was kindly provided for research purposes by Dr. Dieter C. Gruenert (Cardiovascular Research Institute at the University of California, San Francisco, Calif., USA).

2.3. Analytical methods used

2.3.1. GC-FID operating conditions to evaluate the chemical stability of 4-OPA, 4-AMCH, IPOH in the culture medium

The stability of chemicals prepared into the culture medium was analyzed using an Agilent GC 6890 with a liquid injection autosampler. The detection was performed by means of flame ionization detector (FID) at 250 °C. The injector was set-up in split mode at 250 °C, with a split ratio of 1:20. The column used was a J&W HP-5MS, 5% Phenyl Methyl Siloxane, 30 m × 0.25 mm i.d. with 1 μm film thickness (J&W Scientific, USA). The GC oven was programmed as follows: column oven temperature was initially 40 °C; hold for 7.5 min, and increased to 90 °C at the rate of 4 °C/min, and to 300 °C at the rate of 15 °C/min, hold for 2 min. Helium was used as carrier gas at 1.5 mL min^{-1}. Sample was injected by the instrument's autosampler with injection volume of 1 μl. The syringe was cleaned between injections with water and acetonitrile.

The analytical method was calibrated with standard solution prepared in acetonitrile with a concentration ranging from 0.2 to 500 μg mL^{-1}. The test sample solutions (chemicals solubilized into culture medium) were prepared by considering two fetal bovine serum's concentration added to the culture medium: one with 10% and another with 1%.

2.3.2. Samples preparation

The glass vials containing aliquots of chemicals prepared in culture medium or acetonitrile were kept at 37 +/−1 °C, with 5% CO_2 and 95% humidity for 24 h (the same incubator conditions were also used during cell exposure). Before analysing the test sample solution aliquots at given time intervals 1, 2, 3 and 24 h, acetonitrile (v/v, 1:100) was added to both test solutions to precipitate the proteins. Therefore, the sample solution was centrifuged at 10 000 rpm for 10 min in order to eliminate the precipitate. The chromatographic peak areas of the target chemicals solubilized into culture medium was compared to the corresponding peak areas of the standards prepared in acetonitrile.

2.4. In-vitro methods used

2.4.1. Cell culture maintenance and chemical exposure

A549 cell line was grown in RPMI 1640 medium containing 10% FBS and antibiotics (penicillin 100 U mL^{-1} and streptomycin 100 μg mL^{-1}), while 16HBE14o- cell line was maintained in MEM medium with 10% FBS, antibiotics and 1% L-glutamine. Both cell lines were kept at 37 °C, 5% CO_2 in humidified air (relative humidity 95%). The cells were harvested with trypsin/EDTA and seeded as 150 μl/well to a density of 3×10^4 cells mL^{-1} into a 96-well plate and incubated overnight.

Target chemicals were dissolved into the culture medium containing 1% FBS and later diluted by serial dilutions to obtain ten different concentrations ranging from 0.2 to 115 mM (4-OPA), 0.03–17.5 mM (IPOH) and 0.01–5.8 mM (4-AMCH). The highest concentration was determined by the aqueous solubility of chemicals. The cell culture medium from the 96-well plates was aspirated and 150 μl of each dilution was added to the plates in six replicates. Cell negative control (*e.g.* culture medium with cells), blanks (culture medium without cells) were used for the calculations (*e.g.* the blanks were used to correct the absorbance values), while the cell positive control (*e.g.* sodium dodecyl sulphate) was considered to decide whether the experiment can be accepted. The cell cultures were incubated for 24 h and thereafter the NRU assay was carried out to determine cell viability.

2.4.2. Cellular viability by Neutral red uptake assay

According to the NRU protocol (Anon. INVITTOX, 2008, DB-ALM protocol 78), after cell exposure to the target chemicals (24 h), cells were washed with prewarmed PBS (150 μl/well). The washing solution was removed and the neutral red dye was added (100 μl/well) to both 16HBE14o- and A549 cells and incubated for 3 h at 37 °C. Afterwards, the plates were washed with PBS (150 μl/well), followed by the addition of an acetic acid/water/ethanol (1:49:50, v/v/v) solution (150 μl/well). During the incubation with the acetic/water/ethanol solution, the plates were shaken for 10 min. Then, those were allowed to incubate for 30 min at room temperature prior to the measurement of the absorbance at 540 nm.

2.4.3. Inflammatory response measured by cytokine/chemokines assays

Cell culture supernatants, previously collected (step done before washing the cells with prewarmed PBS prior to the addition of neutral red dye), were used to quantify the production of cytokines using a MILLIPLEX MAP Kit (HCYTOMAG-60K, Millipore, Billerica, MA), according to manufacturer's protocol. This approach

allowed for the simultaneous measure of the following human cytokines/chemokines: IL-6, IL-8, TNF-alpha, IL-10 and IL-13. The plate was ran immediately on a Luminex® 100™/200™ platform (Luminex Corporation) with xPONENT 3.1 software. Additionally to the negative (culture medium with and without cells) and positive (lipopolysaccharide: LPS O113:H10) controls, the tested chemicals solubilized into the culture medium were added to the plate to overcome possible chemical's interference with the assay.

2.5. Statistical analysis

At least three independent experiments were performed for each chemical. The cytotoxicity of the tested chemicals evaluated by NRU assay was expressed as the percentage viability (uptake of neutral red dye by the lysosomes) compared with controls in terms of its IC_{50} (the half maximal concentration of a chemical that causes 50% inhibition of growth), which was calculated from the concentration-response curves by linear regression analysis performed with the GraphPad Prism software package v.5.0. Comparison of the cell lines sensitivity to chemicals was made with a two-way Anova test with Dunnett's post-hoc test and an unpaired Student's *t*-test. The level of statistical significance was set to $p < 0.05$ (*e.g.* distinguishing between no observed effect concentration (NOEC) and lowest-observed-effect concentration (LOEC)).

The cytokines/chemokines IL-8, IL-6, TNF-alpha, IL-10 and IL-13 were quantified after the background controls were substracted from the measured fluorescence values. Cytokine/chemokines concentrations in sample were determined with a 5-parameter logistic curve. Final concentrations were calculated from the mean fluorescence intensity and expressed in pg mL^{-1}.

3. Results

3.1. Chemical stability of 4-OPA, 4-AMCH, IPOH in culture medium determined by GC-FID

The stability of the target chemicals over 24 h was tested by solubilising the chemicals in the culture medium containing 1 and 10% of FBS. Based on GC-FID methodology (see Materials and methods), the outcomes concerning the LOPs solubilized into the culture medium showed different stability behaviours over time (see Fig. 1).

As depicted in Fig. 1, 4-OPA dissolved in the culture medium containing 10% FBS, suffered a significant decrease of more than 50% ± 2.9 after 24 h. IPOH significantly decreased around 50% ± 5.58, after 24 h, while the reduction of 4-AMCH was negligible. On the other hand, outcomes from analysis where the compounds were solubilized into the culture medium containing 1% FBS, showed that 4-OPA is slightly degraded, around 6% ± 0.16 after 24 h, while both IPOH and 4-AMCH are the more stable compounds showing no degradation after 24 h.

The higher concentration of serum (*e.g.* 10% FBS) added as cellular nutrient in the cell culture medium showed to influence the chemicals stability after 24 h of their preparation in culture medium, especially for 4-OPA and IPOH. Additional tests on cell culture growth under the selected culture medium conditions showed that the reduction of FBS content from 10% to 1% (the medium with 1% FBS was added to the cells for 24 h—time corresponding to the treatment time) did not influence the viability or the metabolism activity of the cells (data not shown). Therefore, in order to keep as stable as possible the concentrations of 4-OPA, IPOH, 4-AMCH during the selected exposure times of human pulmonary cells to the compounds, the concentration of FBS added to culture medium was 1%.

3.2. Cellular viability analysis of human pulmonary cells treated with 4-OPA, IPOH and 4-AMCH

As can be observed in Fig. 2, a concentration dependent decrease in cell viability was observed for both cell lines (A549 and 16HBE14o-) when treated with 4-OPA and IPOH. Within the concentration range tested, 4-AMCH was found to cause a less cytotoxic effect (approximately 15% of A549 and 50% 16HBE14o- loss in cell viability at 5.8 mM) than the other two chemicals. Based on the curves obtained, the concentration of each chemical where the cell viability was inhibited by 50% was determined by calculating the specific half maximal inhibitory chemical concentration (IC_{50}). The calculated IC_{50} of 4-OPA for A549 cells was 1.6 mM [95% confidence interval (CI 95%): 1.47–1.78] and for

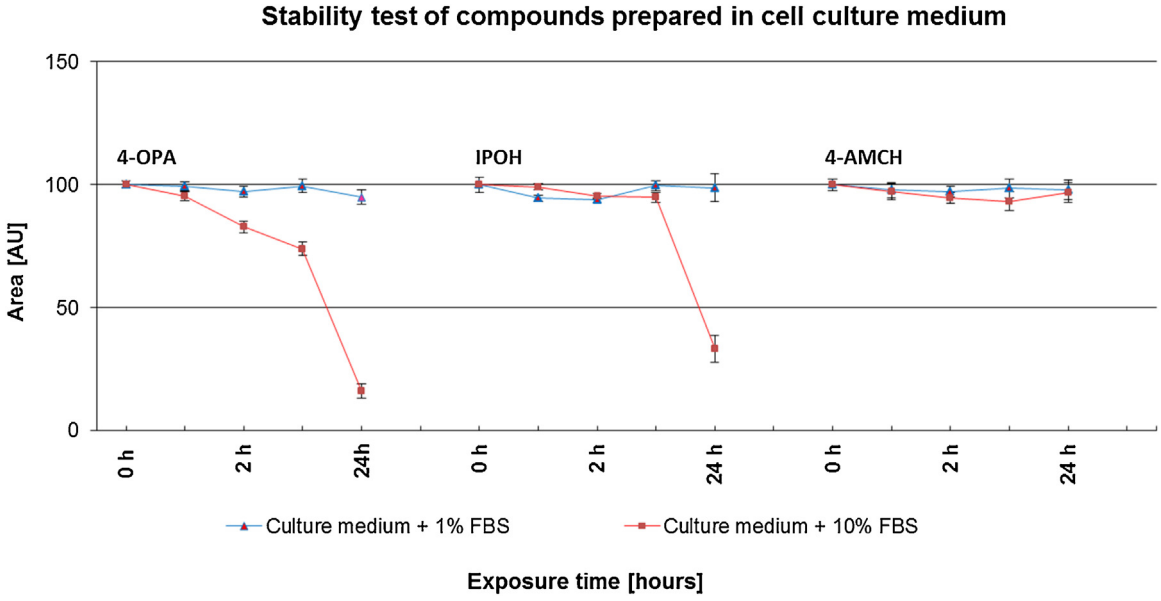


Fig 1. Stability analysis of 4-OPA, 4-AMCH and IPOH solubilized into the culture medium at various concentration of Fetal Bovine Serum (FBS) carried out by GC-FID over time (0, 1, 2, 3, 24 h), (n = 3 ± STD).

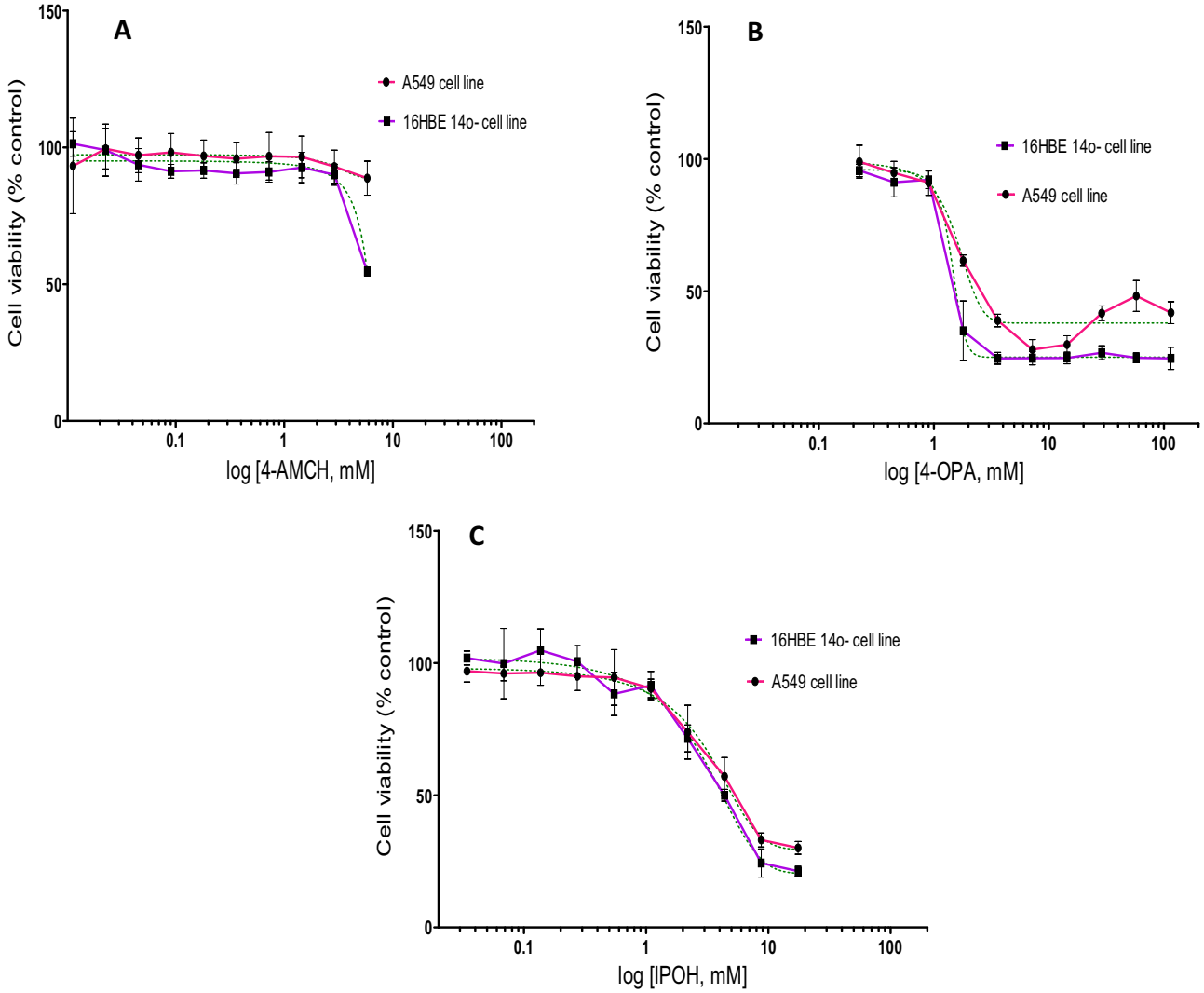


Fig. 2. Concentration-response curves obtained from A549 and 16HBE14o- cells exposed to A. 4-AMCH (0.01–5.8 mM), B. 4-OPA (0.2–115 mM), C. IPOH (0.03–17.5 mM). The dash line represents the trend of 4-parametric sigmoidal curve fit. The results were expressed as percentage (%) of control (untreated cells) ± SD of 3 independent experiments (6 replicates each).

16HBE14o- was 1.45 mM [CI 95%: 1.28–1.64]. The IC_{50} for IPOH in A549 cells was 3.5 mM [CI 95%: 3.04–4.2] while for 16HBE14o- it was found to be 3.4 mM [CI 95%: 2.57–4.09]. However, when their IC_{50} was compared based on their 95% confidence interval, no statistical difference between the two cell lines treated with IPOH was noticed.

Concerning the cellular viability of both cell lines, 16HBE14o- cells showed a more sensitive reaction than A549 cells ($p < 0.005$, one-way analysis of variance (ANOVA) with Tukey's multiple comparison test) to 4-OPA treatment at 28.7, 57.5 and 115 mM. The IC_{50} of 4-AMCH could not be established in the selected concentration range (limitation due to its maximum water solubility of 811 mg L^{-1} at 25 °C).

The lowest observed effect concentration (LOEC) which led to a significant decrease of cells viability ($p < 0.05$) when compared to untreated cells was 0.9 mM and 0.9 mM for A549 and 16HBE14o- cells treated with 4-OPA; 1.1 and 0.6 mM for A549 and 16HBE14o- cells treated with IPOH and 2.9 mM for both cell lines treated with 4-AMCH.

3.3. Inflammation studies of 4-OPA, IPOH and 4-AMCH on human pulmonary cells

Concentrations of the selected chemicals greater than 0.5 mM were found to significantly inhibit the cellular viability ($p < 0.05$). Therefore, three sub-toxic concentrations (1.5, 50 and 500 μM) were further used to evaluate their capacity to induce inflammatory response in A549 and 16HBE14o- cell lines. No response for any of the cytokines/chemokines tested: IL-6, IL-8, TNF-alpha, IL-10 and IL-13 were observed when both cell lines were exposed to 0.5 mM.

As presented in Fig. 3, A549 cells treated with 4-OPA at 1.5 μM expressed up to 1.3-fold higher levels of IL-8 ($p < 0.05$), 1.4-fold higher levels of IL-6 ($p < 0.05$) and 1.7-fold higher levels of TNF-alpha ($p < 0.01$) when compared to the cytokine level determined in untreated cells (negative control). After treatment with 4-OPA at 50 μM, A549 cells released statistically significant levels of IL-6, IL-8 and TNF-alpha (~1.4-fold higher than those of the negative control, $p < 0.05$). 16HBE14o- cells released lower amounts of IL-6, IL-8 and TNF-alpha when exposed to 50 μM of 4-OPA compared to

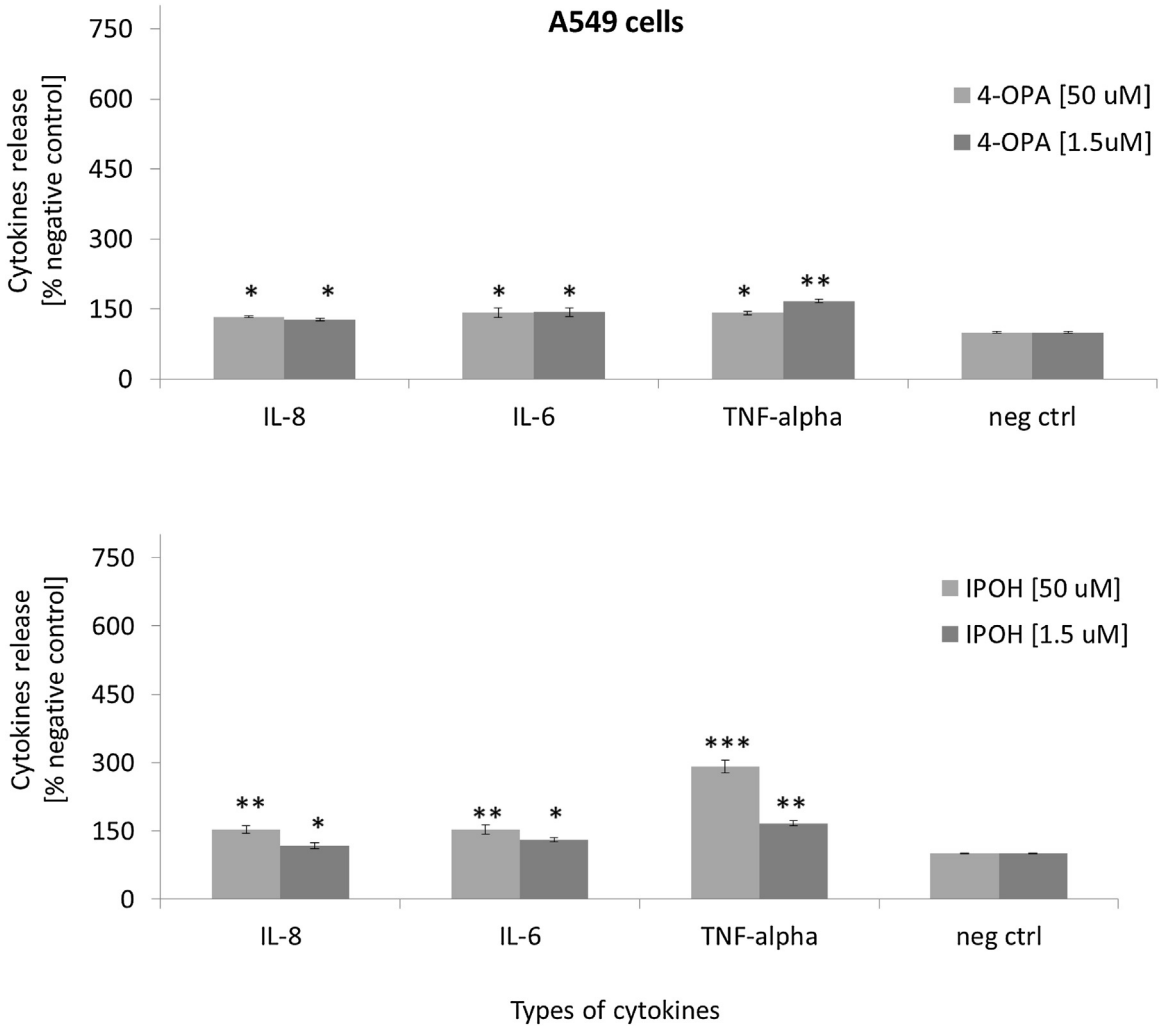


Fig. 3. Total production of various pro-inflammatory cytokines (IL-6, IL-8, TNF-alpha) released by A549 cells after 24-h stimulation with 4-OPA and IPOH at 1.5 and 50 μM. Cells treated with medium (1% FBS) are expressed as "neg ctrl" = negative control (IL-8 = 132 pg mL^{-1}, IL-6 = 81 pg mL^{-1}, TNF-α = 12 pg mL^{-1}) (n = 9 ± SD; *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$).

the amounts observed in the negative control levels (cells untreated).

On the other hand, a concentration-dependent effect was observed in case of IPOH (see Fig. 3). The levels of IL-6 released by A549 cells were 1.3-times at 1.5 μM IPOH ($p < 0.05$) and 1.5-times at 50 μM IPOH higher than the amount found in untreated cells ($p < 0.01$). A similar behaviour was observed in 16HBE14o- cells: as IPOH concentration increased, the amount of IL-8 was increasing from 2.3-times to 2.8-times ($p < 0.001$); levels of IL-6 were found to be approximately 3- and 7-fold change higher than the levels measured in cell controls; and the amount of TNF-alpha calculated was 1.4 ($p < 0.05$) and 1.7-times higher ($p < 0.01$) (see Fig. 4).

As shown in Table 2, in both cell lines (A549 and 16HBE14o-), 4-AMCH significantly increased the production of TNF-alpha (1.5-times, $p < 0.05$) when treatment was done at the higher concentration tested (50 μM). Enhanced production of IL-6 and IL-8 (2.5 and 2-times, respectively) was observed in 16HBE14o- cells treated with 1.5 μM 4-AMCH.

Observing the variations in some of the pro-inflammatory cytokines levels, it was decided to further investigate anti-inflammatory mediators such as IL-10, IL-13. Therefore, ratio trends of the pro-inflammatory cytokines to anti-inflammatory cytokines were calculated for all tested compounds and compared as follows: IL-8/IL-13, IL-8/IL-10 TNF-alpha/IL-10, TNF-alpha/IL-13, IL-6/IL-10, IL-6/IL-13. A ratio increase (more than 1) of the pro-inflammatory versus anti-inflammatory cytokines mentioned above was considered to represent an inflammatory response, as dictated by the balance between pro and anti-inflammatory biomarkers (Gogos et al., 2000).

As can be seen in Fig. 5, a marked increase in the fold change of IL-8 versus IL-10 (~8.5-times) was found in the bronchial cells (16HBE14o-) exposed to IPOH compared with the fold-change ratio of same cytokines released by the A549 cells (IL-8/IL-10 ratio was 1.5). In contrast, both TNF-alpha/IL-10, TNF-alpha/IL-13 fold changes were found to be at higher levels (3-times) in A549 cells (see Table 3) than (2- and 1.5-times, respectively) in 16HBE14o- cells (see Table 4). The data on the fold-change ratio calculated for 4-AMCH show that none of the selected pro-inflammatory versus anti-inflammatory cytokines was secreted in bronchial cells. Moreover, there was no statistically significant changes in the ratio of pro versus anti-inflammatory cytokines noted when bronchial cells were incubated with the tested compounds at 1.5 μM compared to the negative control.

4. Discussion

In the present study, the potential toxic effects of IPOH, 4-AMCH, 4-OPA regarding cell viability and inflammation were

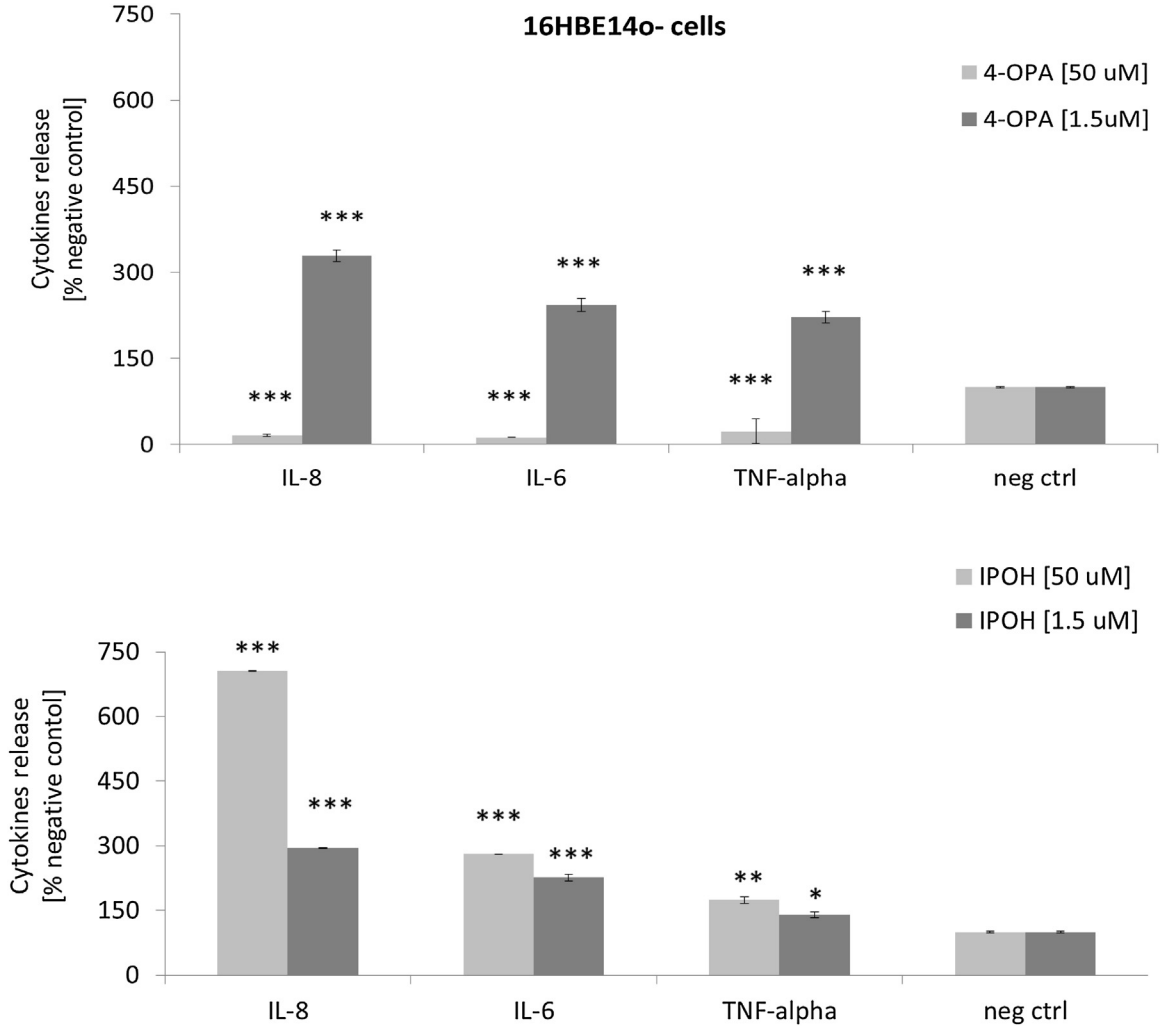


Fig. 4. Total production of various pro-inflammatory cytokines (IL-6, IL-8, TNF-alpha) released by 16HBE14o- cells after 24-h stimulation with 4-OPA and IPOH at 1.5 and 50 μM. Cells treated with medium (1% FBS) are expressed as "neg ctrl" = negative control (IL-8 = 161.7 pg mL^{-1}, IL-6 = 59.5 pg mL^{-1}, TNF-α = 22.5 pg mL^{-1}) (n = 9 ± SD; *p < 0.05, **p < 0.01, ***p < 0.001).

investigated at the cellular level by exposing representative human cell lines of the pulmonary system. The chemicals selected in this *in vitro* work are oxidation reaction products of terpenes such as limonene, [4-OPA (Forester and Wells, 2009), IPOH (Hakola et al., 1994), 4-AMCH (Hakola et al., 1994)], α-terpineol [4-OPA (Forester and Wells, 2009)], geraniol [4-OPA (Forester and Wells, 2009)]. Per se, they might be responsible for certain adverse health effects linked to terpene-ozone exposure such as: decrease in respiratory frequency, airflow limitations (Wolkoff et al., 2013), skin irritation and sensitization (Anderson et al., 2010).

Although 4-OPA, IPOH and 4-AMCH were studied *in vivo* by murine model (Wolkoff et al., 2013), much less is known about their potential human lung toxicity. Up to our knowledge, only one *in vitro* study investigated the biological effects (in terms of inflammatory potency) of 4-OPA on A549 cells (Anderson et al., 2010).

To accurately assess the potential public health impacts of exposure to realistic levels of 4-oxopentanal (4-OPA), 3-isopropenyl-6-oxoheptanal (IPOH) and 4-acetyl-1-methylcyclohexene (4-AMCH), the concentrations in various micro-environments where human exposure can take place must be well-known. At present, these data are scarcely available. The lowest concentration reported in the literature regarding 4-OPA was measured in forest environment (around 0.18 μg m^{-3}) (Matsunaga et al., 2004); measured in European offices, IPOH was found to be in the order of 0.4 μg m^{-3}, while 4-AMCH was 0.9 μg m^{-3} (Nørgaard et al., 2014).

As it is challenging to convert airborne concentrations to *in vitro* medium concentrations that are needed for the *in vitro* exposure

Table 2
Summary of fold changes calculated for pro- and anti-inflammatory markers released from A549 and 16HBE14o- cells treated with the selected chemicals (4-OPA, IPOH, 4-AMCH) at different concentrations (1.5 and 50 μM). The values added in bold represent a fold-change decrease (down-regulation) of the investigated inflammatory markers.

Cell lines	Chemical	Conc.[μM]	Cytokines/chemokines (fold changes)				
			IL-8	IL-6	TNF-α	IL-10	IL-13
A549	IPOH	1.5	1.2	1.3	1.7	1	3
		50	1.5	1.5	2.9	1	1
	4-OPA	1.5	1.3	1.4	1.7	1	4
		50	1.3	1.4	1.4	1	2.7
	4-AMCH	1.5	1.2	1.3	**8.5**	1	4.8
		50	1.2	1.2	1.5	1	1
16HBE14o-	IPOH	1.5	2.3	3	1.4	4.2	2.6
		50	2.8	7	1.7	**1.2**	1
	4-OPA	1.5	2.4	3.3	2.2	3.6	7.3
		50	**6.1**	**7.9**	**4.4**	11.6	8.6
	4-AMCH	1.5	2	2.5	1	5.6	8
		50	**7.1**	**4.5**	1.5	1.6	4.3

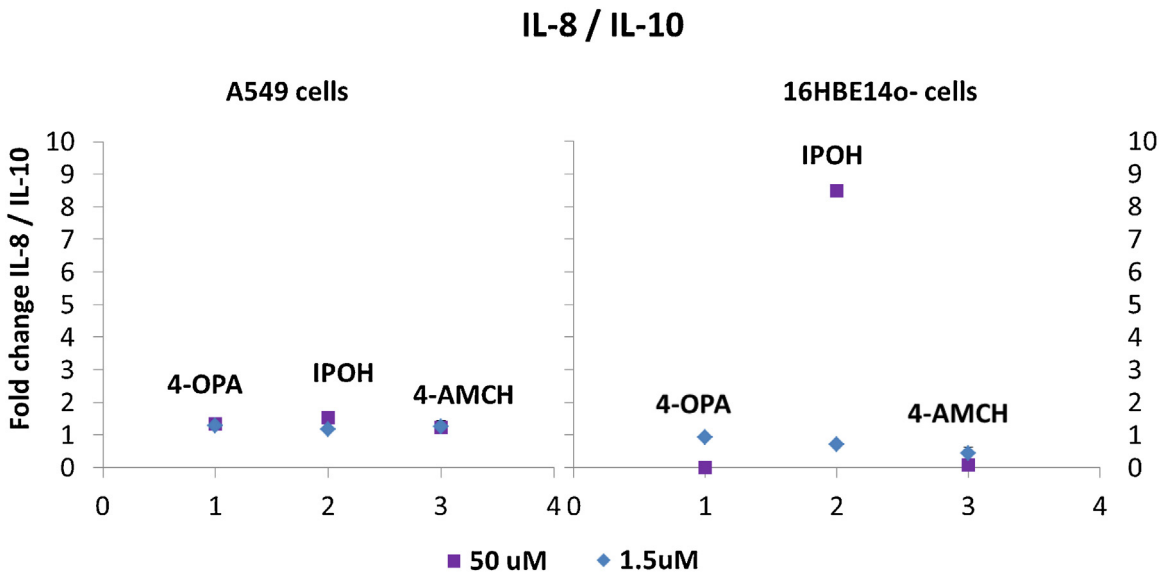


Fig. 5. Fold change of the pro-inflammatory cytokine IL-8 and anti-inflammatory cytokine IL-10 ratio for both A549 and 16HBE14o- cell lines treated with 4-OPA, IPOH, 4-AMCH at 1.5 and 50 μM, in comparison to the corresponding basal ratio of non-exposed cells (negative control).

system that was used (liquid-liquid exposure), a large range of concentrations was tested, starting with the maximum chemical's solubility in the cellular medium and diluting stepwise. This resulted in concentration ranges of 0.2–115 mM (4-OPA), 0.03–17.5 mM (IPOH) and 0.01–5.8 mM (4-AMCH), which were tested on both bronchial epithelial (16HBE14o-), alveolar epithelial (A549) cell lines. The mentioned cell lines were chosen to be study as they are important producers of cytokines—main proteins in lung defence and inflammation (Stadnyk, 1994) and have a central role in the pathophysiology of asthma (being airway epithelial cells, represent the first line barrier of defence).

In vitro studies require a thorough characterization of chemicals stability in the stock solution and/or culture medium over cells' exposure time, since interactions between serum proteins and chemicals can modify, diminish or invalidate the potentially adverse effects of chemicals. Furthermore, it is also important to underline that the nominal concentration in culture medium does not necessarily correspond to the real concentration to which the cells are exposed (Kramer et al., 2015). GC-FID analysis showed (see Fig. 1) that some of the chemicals solubilized in cellular medium containing 10% FBS were unstable over exposure time, therefore experiments were carried out in the presence of only 1% FBS (serum protein's concentration that did not alter the cellular viability). The observed reduction of the tested chemicals that occurs under high serum conditions (*e.g.* 10% FBS) might be caused by the interaction of the tested chemicals – classified as aldehydes – and the residual amino (NH_2) groups on the protein molecules present in the FBS. This reaction of the aldehyde with a NH_2 group on the protein molecule leads to the formation of a Schiff base (*e.g.* a biologically important Schiff base, rhodopsin found in the visual pigment is formed from the reaction of the aldehyde 11-cis-retinal with the amino group of lysine, opsin) (Berg et al., 2002).

The selection of the pro- and anti-inflammatory cytokines investigated in the present study takes into account their key role in pulmonary diseases. Pro-inflammatory cytokines have been postulated to be linked to asthma, pathogenesis of bronchiolitis, in particular IL-6 and IL-8 (Rincon and Irvin, 2012; Yalcin et al., 2012). TNF-alpha has been identified as a key element in various pulmonary diseases such as acute lung injury, asthma, chronic bronchitis (Matera et al., 2010; Antoniu et al., 2008; Mukhopadhyay et al., 2006). *In vivo* studies found that high levels of TNF-alpha causes necrosis in mice (Miyazaki et al., 1995) or induces severe pro-inflammatory reactions (Mukhopadhyay et al., 2006). The existing literature on anti-inflammatory cytokines has suggested that the increased release of IL-10 (a potent anti-inflammatory cytokine) contributed to the reduction of TNF-alpha release in alveolar cells (Armstrong et al., 1996) or decreased production of IL-6 in macrophage (Avdiushko et al., 2001). IL-13 was demonstrated to have a dual role: as pro-inflammatory cytokine, which plays an important role in allergic disease, but it

Table 3
Summary of pro- and anti-inflammatory ratios for A549 cells treated with 4-OPA, IPOH, 4-AMCH at different concentrations (1.5 and 50 μM). The values added in bold represent a fold-change decrease (down-regulation) of the investigated inflammatory markers.

A549 cells						
	4-OPA [uM]		IPOH [uM]		4-AMCH [uM]	
	1.5	50	1.5	50	1.5	50
IL-8/IL-10	1.3	1.3	1.2	1.5	1.3	1.2
IL-8/IL-13	**3**	2	2.6	1.5	3.9	1.2
IL-6/IL-10	1.4	1.4	1.3	1.5	1.3	1.2
IL-6/IL-13	**2.8**	1.8	2.3	1.5	3.8	1.2
TNF-α/IL-10	1.7	1.4	1.7	3	**8.5**	1.6
TNF-α/IL-13	**2.4**	1.9	1.8	3	**4.1**	1.6

Table 4
Summary of pro- and anti-inflammatory ratios for 16HBE14o- cells treated with 4-OPA, IPOH, 4-AMCH at different concentrations (1.5 and 50 μM). The values added in bold represent a fold-change decrease (down-regulation) of the investigated inflammatory markers.

16HBE14o- cells						
	4-OPA [uM]		IPOH [uM]		4-AMCH [uM]	
	1.5	50	1.5	50	1.5	50
IL-8/IL-10	**1.1**	**71.1**	**1.4**	8.5	**2.3**	**11**
IL-8/IL-13	**2.2**	**52.8**	**1**	6.3	**3.2**	**30.2**
IL-6/IL-10	**1.5**	**92.3**	**1.8**	3.4	**2.8**	**7.1**
IL-6/IL-13	**3**	**68.5**	1.2	2.5	**4**	**19.5**
TNF-α/IL-10	**1.6**	**51.1**	**3**	2	**5.5**	1
TNF-α/IL-13	**3.2**	**37.9**	**1.9**	1.5	**7.9**	**2.7**

has also been recognized to have anti-inflammatory properties by suppressing cytotoxic and inflammatory functions of monocytes and macrophages (Cavaillon, 2001) or by limiting the production of TNF-alpha-induced IL-8 in a whole blood assay (Marie et al., 1996).

Exposure of bronchial cells to high concentrations levels of 4-OPA [50 μM] showed a down-regulation of IL-6 (6.1-fold change) and IL-8 (7.9-fold change) when compared to untreated cells. On the other hand, at the same concentration as 4-OPA, IPOH induced an increase of both IL-6 and IL-8 secretion in bronchial cells (7-fold change and 2.8-fold change respectively) which indicates that the deleterious effect of IPOH is higher in the bronchial cells than in the alveolar cells, where IL-6 and IL-8 levels were increasing by 1.5-fold change. A similar behaviour [deleterious effect higher in bronchial (IL-8 = 2.3- and IL-6 = 3-fold change) than alveolar (IL-8 = 1.2- and IL-6 = 1.3-fold change) cells] was observed at the lowest tested concentration for IPOH [1.5 μM]. Concerning the inflammatory effects of 4-AMCH on bronchial cells, only at lowest tested concentration [1.5 μM], both IL-6 and IL-8 levels were up-regulated (∼2-fold change higher than the cells untreated), while at higher concentration [50 μM] the effects observed were the opposite (*e.g.* both IL-6 and IL-8 were approx. 7 and 5-times down-regulated). On the other hand, 4-AMCH [50 μM] induced increased levels of TNF-alpha in A549 cells.

In 16HBE14o- cells treated with IPOH at 50 μM, IL-8/IL-10 ratio was higher than IL-8/IL-13 (∼1.4-times, $p < 0.05$), indicating that IL-10 did not inhibit IL-8 secretion as IL-13 did. Although IL-10 is considered to have anti-inflammatory functions, clinical observations revealed that IL-10 have also pro-inflammatory potential (*e.g.* an increased production trend of IL-8 was observed upon IL-10 treatment) (Cooper et al., 2000). Therefore, it might be that IL-10 played its pro-inflammatory role while IL-13 served as an anti-inflammatory indicator (Cavaillon, 2001; Marie et al., 1996). Under our experimental conditions, the ratios of pro-inflammatory TNF-alpha, IL-6 cytokines versus IL-10, IL-13 anti-inflammatory were found to be higher in A549 cells treated with IPOH [50 μM] when compared to 16HBE14o- cells. Thus, the cellular inflammatory response to IPOH depends on cell type and concentration. In summary, the findings of this study regarding the potential inflammatory capacity of the selected chemicals suggest that the calculated ratio between pro- and anti-inflammatory cytokines caused a relevant increase in the release of inflammatory response in human lung cells.

Results of the *in vivo* study carried out by Anderson et al., showed no significant changes in IL-10 amount when mice were exposed to 4-OPA at 1.97 mM (tested on the mouse ear for dermal sensitisation) or at 0.08 mM (pulmonary exposure) (Anderson et al., 2012). Our results concerning the expression of IL-10 in A549 cells when exposed to concentrations covering the range from 0 to 0.5 mM 4-OPA showed no statistically significant increase amount of IL-10.

In one *in vitro* study, A549 exposed to a gas phase containing 65 ppm 4-OPA released significantly increased levels of IL-6 (1059 pg mL^{-1}, at 12 h), IL-8 (400 pg mL^{-1}, at 24 h) and TNF-alpha (10 pg mL^{-1}, at 24 h) (Anderson et al., 2010). As showed in Results section, 4-OPA tested at a low concentration [1.5 μM], significantly altered all the selected pro-inflammatory cytokines as follows: IL-6 (∼116 pg mL^{-1}, at 24 h), IL-8 (∼167 pg mL^{-1}, at 24 h), and TNF-alpha (∼20.1 pg mL^{-1}, at 24 h). At a higher concentration of 4-OPA [50 μM], after 24 h, the amount of IL-6 was not statistically different when compared to the IL-6 amount obtained after 4-OPA treatment at 1.5 μM, while TNF-alpha levels slightly decreased (∼17.2 pg mL^{-1}, $p < 0.05$).

Among the tested compounds, IPOH has proven to have the strongest potency for induction of inflammatory cytokines IL-6 and IL-8 (*e.g.* approximately 200-fold change increase of IL-6 for 16HBE14o- cells stimulated with IPOH when compared to 16HBE14o- cells treated with 4-OPA) whereas 4-OPA demonstrated the strongest cell viability destruction of A549 cells with a IC_{50} at 1.6 mM (when compared to IC_{50} IPOH = 3.5 mM and IC_{50} 4-AMCH could not be established).

In conclusion, the findings of the present study demonstrate, for the first time, to the best of our knowledge, that at 50 μM both 4-OPA and 4-AMCH inhibited the expression of IL-6, IL-8, TNF-α in 16HBE14o- cells, but not in A549 cells. On the other hand, IPOH [50 μM] was stimulating IL-6, IL-8 and TNF-α release from both 16HBE14o- and A549 cell lines, suggesting that IPOH is likely to contribute to the pathogenesis of human airway inflammatory disorders. The results regarding the inflammatory capacity of IPOH agree reasonably well with the recent observations made by Wolkoff et al. regarding the awareness on IPOH (based on his *in vivo* results, IPOH might cause sensory irritation) (Wolkoff et al., 2013).

Disclaimers

The content of this article is under the responsibility of the authors and may not in any circumstances be regarded as stating an official position of the European Commission.

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