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Molecular targets of chloropicrin in human airway epithelial cells

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Molecular targets of chloropicrin in human airway epithelial cells

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Abstract

Chloropicrin is a vaporizing, irritating compound that causes complications in the respiratory system when inhaled. In this study, we examined the effects of exposure to chloropicrin for 24 h on ultrastructure and global gene expression in primary human bronchial epithelial cells. The treatment increased the number of round and shrunken cells, which detached from culture plates more readily than the untreated control cells. Transmission electron microscopy revealed some swollen mitochondria and the appearance of autophagy/lysosome type of vacuoles in the treated cells. However, the main alteration in the ultrastructure of the treated cells was the presence of aggregated and slightly deformed cytoskeleton structures. Furthermore, confocal microscopy and immunoblotting indicated that cytoskeletal β-tubulin protein is a probable target of chloropicrin exposure. Ingenuity Pathway Analysis (IPA) of differentially expressed microarray data (fold change > ± 2 compared to controls considered) revealed that the top molecular functions were cell growth and proliferation. The main enriched top canonical pathways identified by IPA were associated with EIF2-signalling, protein ubiquitination pathway, glycolysis and mitochondrial dysfunction. Furthermore, the main upstream regulators and their target genes were involved in cell growth and proliferation and cytoskeletal organization. The alterations found here can be the core components of toxicity involved in the lung complications after chloropicrin exposure.

Key words: Bronchial epithelial cells, aggregates, β-tubulin, transmission electron microscopy, confocal microscopy, microarray
Abbreviations: DAPI, 4’,6-Diamide-2’-phenylindole dihydrochloride; ECACC, European Collection of Cell Culture; EIF2, eukaryotic initiation factor 2; HBEpC, human bronchial epithelial cells; IPA, Ingenuity Pathway Analysis; ROS, reactive oxygen species.
**Introduction**

Chloropicrin (CCl₃NO₂) is an oily, easily vaporized liquid that has strong irritating properties. It is used for disinfecting grains, synthesis of crystal violet and as a pesticide to fumigate soil against insects and other organisms (EPA-report, 2008; Ruzo, 2006). Small amounts of chloropicrin can also be formed during chlorination of water (Bond, et al. 2014). The main source for human exposure is occupational i.e. when chloropicrin is being manufactured and utilized. A high acute exposure can also occur due to accidents or intentional release of chloropicrin into the environment. Exposure to a low concentration of chloropicrin (< 1 ppm) in the air causes irritation in the eyes and respiratory system. Because of its volatility, the main route of human exposure to chloropicrin is inhalation. Consequently, respiratory difficulties; cough, dyspnoea, burning, chest pain and inflammation have been reported after the exposure. Higher concentrations damage the respiratory tract and lead to emphysema and life-threatening oedema (EPA-report, 2008; Gonmori, et al. 1987). In addition to the respiratory tract, chloropicrin is toxic in experimental animals in organs such as kidney, muscles and stomach (EPA-report, 2008). There is evidence suggesting that its toxicity involves oxidative stress by increasing the amounts of reactive oxygen species (ROS), depleting cellular glutathione as well as interacting with the thiol-groups in amino acids (Pesonen, et al. 2014; Sparks, et al. 1997). However, the overall mechanism(s) responsible for the toxicity of chloropicrin is not well understood.

There are an increasing number of studies revealing that stress conditions caused by environmental chemicals alter the integrity of the cytoskeleton. These changes may affect cell signalling and gene expression, and thereby contribute to the pathways leading to the
development of pathological, toxic conditions in tissues (Go, et al. 2013; Kanda, et al. 2014; Toivola and Eriksson, 1999; Toivola, et al. 2010). Cytoskeleton integrity is dependent on the dynamic network of the filament systems that consist of three main components; microtubules, intermediate filaments and microfilaments. Microtubules are formed from polymerized tubulin proteins whereas microfilaments are made up of polymerized actin proteins. The proteins in the intermediate filaments are more heterogeneous and expressed in a cell type specific manners e.g. in epithelial cells, they consist of keratins (Pollard, et al. 2008). The cytoskeleton and its associated proteins are very abundant in many cell types (Pollard, et al. 2008) and thus, likely targets to chemical exposures.

Our previous studies (Pesonen, et al. 2012; 2014) have shown that chloropicrin increases oxidative stress, elevates the expression of the mitogen activated protein kinase (ERK1/2) and of proteins associated with endoplasmic reticulum stress. Furthermore, chloropicrin has been shown to increase cell cycle regulating proteins (p53, p27 and p21), trigger the G2/M-phase arrest and to increase a number of autophagy/lysosome vacuoles in human cell lines (Pesonen, et al. 2015). Since chloropicrin inhalation causes primary damage to the epithelium of the respiratory tract, in this study we have used primary human bronchial epithelial cells as the experimental model. These cells are the first line of defence against inhaled volatile irritants like chloropicrin. The main aim was to identify early responses and molecular pathways, which may underlie chloropicrin toxicity. The knowledge of early responses in the epithelial cells is valuable in the development of biomarkers and antidote to combat acute accidental exposure to chloropicrin. Here, we have used microscopy, microarray and immunoblotting to detect potential targets of chloropicrin in human bronchial epithelial cell.
2. Material and Methods

Caution: Chloropicrin is a reactive, volatile, and toxic chemical. It must be handled carefully using protective gloves and glasses, with all procedures conducted in a laminar hood in order to avoid contamination.

2.1. Chemicals

Human primary bronchial epithelial cells (HBEpC) and Bronchial Epithelial Cell Growth Medium were obtained from European Collection of Cell Cultures (ECACC, Salisbury, UK). Chloropicrin (CCl3NO2, CAS#: 76-06-2) was from Sigma-Aldrich (Helsinki, Finland) and DAPI from Thermo Fisher Scientific Inc. (Vantaa, Finland). Epon (LX-112-recin) was purchased from Ladd Research Industries Inc. (Williston, USA) and Uranyl acetate from Electron Microscopy Sciences (Hatfield, USA). Protein assay reagents were obtained from BioRad Laboratories Inc. (Espoo, Finland). Primary antibodies (anti-tubulin-β antibody and anti-β-actin antibody) were from Cell Signaling Technology (Danvers MA, USA) and the secondary antibody, Texas Read anti-rabbit IgG (H+L) from Vector Laboratories Inc. (Burlingame, USA). ECL primer Western blotting detection reagent was obtained from Fisher Scientific (Vantaa, Finland) and PVDF-membrane from Merck-Millipore (Espoo, Finland). ECL™ anti-mouse IgG HRP-labelled antibody was from Amersham BioSciences (Buckinghamshire, UK) and anti-rabbit IgG (goat) peroxidase conjugated antibody from CalbioChem (Darmstadt, Germany). The 10 cm culture plates were purchased from Sarstedt inc. (Newton, USA) and Ibidi microscopy chambers from Ibidi GmBH (Martinsried, Germany). All other chemicals used were of analytical grade.

2.2. Cell Culture and Treatment
HBEpC-cells were cryopreserved at first passage and grown in bronchial/tracheal epithelial cell growth medium at 37°C in a humidified incubator (with 5 % CO₂ and 95 % air) according to the instructions provided by ECACC. The cells were kept in culture for 2-3 days before treating them with chloropicrin (10 μM and 40 μM) for 24 h. The used concentrations of chloropicrin were chosen basing on our previous cytotoxicity assay with HBEpC cells (Pesonen et al. 2015). According to that study the low concentration (10 μM) did not have effect on cell viability and the higher concentration (40 μM) was near but still under the EC₅₀-value of chloropicrin in HBEpC cells. The vehicle control (0.1% DMSO) did not have any effects on the measured parameters when compared to the medium without DMSO.

2.3. Transmission Electron Microscopy (TEM)

In the TEM analysis (JEM-2100F, from Jeol, Japan) control and treated cells were fixed with 2.5 % glutaraldehyde in phosphate buffer (pH 7.4) and post-fixed in 1 % osmium tetraoxide (OsO₄) for 3 h. Thereafter the cells were dehydrated with increasing concentrations of ethanol and embedded in Epon (LX-112) blocks. The blocks were sectioned into ultrathin slices and double stained with uranyl acetate and lead citrate.

2.4. Immunofluorescence and Confocal Microscopy

HBEpC cells grown on Ibidi plates were rinsed with DPBS and fixed with 4% paraformaldehyde at room temperature for 60 min. After fixation, the cells were permeabilized with 0.1% Triton X-100 in DPBS for 10 min and blocked with 1 % BSA in DPBS for 30 min. Thereafter, the cells were incubated at 4°C over night with the primary antibody, human anti-tubulin-β antibody, diluted 1:200 in 1 % BSA. Next morning plates were rinsed and treated for one hour at room temperature with the secondary anti-rabbit
antibody (diluted 1:150). Thereafter, nuclei were stained with DAPI (1μg/ml) at 37°C for 15 min and images were visualized using a ZeissAxio observer inverted microscopy equipped with a Zeiss LSM 800 confocal module (Carl Zeiss microimaging GmbH, Jena, Germany). Three independent treatments with 3 replicates were performed.

2.5. Electrophoresis and immunoblotting analysis

The whole cell fractions were used in immunoblotting. Preparing of cell fractions has been described previously (Pesonen et al. 2014). Protein concentrations of the fractions were measured using Bradford method. Equal amount of cell proteins (10 μg) were loaded onto 12 % acrylamide gel and separated by electrophoresis at 200V for 60 min (BioRad Mini-protean vertical electrophoresis) and transferred to PVDF-membrane using Trans-Blot semi-dry transfer cell. After blocking for 2 h in 5% non-fat cow milk-TBS, the membranes were incubated overnight with primary antibodies (anti-β-tubulin antibody, diluted 1:1000 and anti-β-actin antibody, diluted 1:200000) at 4 °C. Thereafter the membranes were washed and treated with secondary antibodies (diluted 1:2000) for 1 h at room temperature. Protein bands were visualized with ECL+ Plus system (immunoblotting detection system, Amersham BioSciences), according to the manufacturer’s instructions. Densitometric analysis of protein bands was carried out using QuantityOne®-program (1-D Analysis Software, version, BioRad Laboratories Inc. USA) and the data were normalised by the loading control, β-actin. The results are expressed as a fold of the control values. Three independent experiments for each protein were performed.

2.6. Extraction of total RNA and DNA microarray analysis
After the treatment, the cells were washed with PBS-solution and the total RNA was extracted using TRI-reagent as described previously (Storvik et al. 2011). The extracted RNA was stored at -80 °C before analysis. Three replicates were used for the further study. The microarray work was carried out at the Core Facility of the Estonian Genome Center, University of Tartu (an Illumina CSPro lab). The procedure has been described previously by Pesonen et al. (2015). In this study, we present microarray results at 24 h of exposure to chloropicrin. The other part of the study i.e. microarray results at 6 and 48 h after the exposures were published previously (Pesonen et al. 2015). The microarray data were pre-processed and normalized to median with Chipster software (CSC, Espoo, Finland). The differentially expressed gene sets were selected based on filtering to detect a fold-change > ± 2 as compared to the respective controls. The data sets of differentially expressed genes were examined according to their functional enrichment in gene networks, top pathways and upstream regulators using the Ingenuity Pathway Analysis (IPA, Qiagen CA, USA). A real time qPCR assay of six selected genes (ATF3, CRYAB, HMOX1, HSPA6, MYLIP, ODC1) was performed at 6 hours after the exposure to validate microarray results. The validation showed a good agreement between DNA-microarray data and the mRNA levels. The qPCR procedure and validation results were presented previously by Pesonen, et al. (2015).

3. Results

3.1. Chloropicrin modifies cell structure

Chloropicrin treatment increased the number of round and shrunken cells that detached more readily from culture plates and lost their cell-to-cell contacts in comparison with untreated control cells (Fig. 1). Transmission electron microscopy revealed the appearance of
deformed, slightly aggregated and fragmented cytoskeletal structures in the treated cells (Fig. 2). These were not seen in control cells. There were also some swollen mitochondria in the treated cells but generally mitochondria remained intact (data not shown). In addition, the treatment elevated an electron dense autophagy/lysosome type of vacuoles indicative of increased degradation of damaged cell components (Fig. 2).

Since our previous study had shown that chloropicrin-evokes G2/M cell cycle arrest (Pesonen, et al. 2015), which could result from damage to tubulin proteins, we next investigated β-tubulin using immunofluorescence. Beta tubulin together with α-tubulin are the principal component of microtubules. In control cells, the β-tubulin immunofluorescence labelling was evenly distributed in the cytoplasm (Fig. 3). Instead, the immunofluorescence in the treated cells was irregularly distributed and there was also a retraction of tubulin filaments (Fig. 3). Some of the cells, particularly those treated with the higher concentration of chloropicrin, displayed only weak immunofluorescence, partly as punctuated spots, suggesting disruption of tubulin proteins. This was further indicated by immunoblotting that showed reduction of β-tubulin band compared to the band of control cells at 24 h of exposure (Fig. 3).

3.2. Chloropicrin affects expression of transcripts

To identify functional categories, significant pathways and top up-stream regulators in the differentially expressed transcripts of our microarray data set, we performed an IPA-pathway analysis. The top molecular function, enriched by IPA (Table 1), was the cellular growth and proliferation and this was noted with both exposure concentrations. Several transcripts, which were enriched in the top 10 network pathways, encoded cytoskeletal proteins (Fig. 4). The transcripts encoding tubulin and heat shock proteins were up-
regulated. In contrast to tubulins, the transcripts encoding cytokeratin, actin and some proteins associated with polymerization of actin (e.g. CFL1, Wdr1, LIMA1) were down-regulated (Fig. 4) suggesting that the response of these genes to chloropicrin exposure is different from that of tubulins.

The main top canonical pathway enriched according to the IPA-software (Table 1) was the eukaryotic initiation factor 2 (EIF2) signalling with both exposure concentrations. EIF2 is involved in initiation of translation. In this study, this pathway contained only one up-regulated transcript (EIF4A), the other transcripts were down-regulated (e.g. eIF3, 40S, 60S, PABP) suggesting that chloropicrin interferes with translation. The other main top canonical pathways (Table 1) were protein ubiquitination, mitochondrial dysfunction and glycolysis. The mitochondrial dysfunction consisted of many down-regulated transcripts encoding proteins participating in the electron transport chain, complexes I and III-IV (e.g. NDUF, UQCR, COX5B, COX6B1) and several ATP synthases. The top upstream regulator, enriched by the treatment (Table 1) was a member of transforming growth factor-β1 (TGFβ1) signalling. This growth factor is a multifunctional peptide regulating growth, differentiation and repair in many cell types. Here, TGFβ1 signalling was inactivated and its target genes were among those involved in cytoskeletal organization, cell adhesion, cell migration and anti-oxidation (e.g. TPM2, THBS1, SDC1, MAP2K1, TXNIP). The other main top upstream-regulators were the activated proto-oncogenes MYC and NMYC and their target genes were involved in cellular growth, proliferation, ribosomal protein, cytoskeletal tubulins and cell cycle (e.g. EGR1, FOS, RPL18A, TUBB, TUBA1B, CDKN1A).

4. Discussion
In this study, we describe alterations in the cellular ultrastructure and global gene expression patterns in primary human bronchial epithelial cells after exposure to chloropicrin. We observed deformed and aggregated cytoskeleton structures and weakening of cell attachment after cells were exposed to chloropicrin. These alterations were accompanied by changes in various transcripts related to cellular growth and cytoskeleton and to important cellular metabolic processes such as translation, protein ubiquitination, glycolysis and mitochondrial dysfunction. Of these the particular interest are the mitochondrial dysfunction and cytoskeleton because mitochondria are important for energy balance and cytoskeleton plays an important role in maintenance of cell shape and intracellular organization. Cytoskeleton is also involved in many cellular processes such as cell division, organelle transport, motility, signal transduction, growth and development (Aung, et al. 2015; Galluzzi, et al. 2014; Pollard, et al. 2008).

The results here show that chloropicrin alters the integrity of cytoskeletal β-tubulin in human bronchial epithelial cells. Several mechanisms have previously been proposed to be involved in the alterations of cytoskeleton integrity by exposure to environmental contaminants. These include specific inhibition of phosphatases (PP1A, PP2A) resulting in increased phosphorylation of cytoskeletal proteins, disturbance of cellular calcium status, ATP-depletion, as well as thiol-oxidation and other oxidative stress related causes (Eriksson, et al. 1992; Gonsebatt, et al. 2007; Kanda, et al. 2014; Wickstrom, et al. 1995). In this study, the most probable cause for the chloropicrin-evoked alterations to the cytoskeleton and cellular structures was the formation of reactive oxidation products. Our previous studies clearly revealed that chloropicrin depletes cellular glutathione and increases the formation of disulphide bridges between thiol-groups (SH) in cysteine residues (Pesonen, et al. 2014).
Thus, the deformed and aggregated cytoskeletal structures seen in the TEM-images may well be oxidised proteins and disulphide bridges formed between thiol-containing proteins.

The initial toxicity in this study possibly resulted from the reactivity of chlorine, because chlorine is known to damage respiratory epithelium by oxidative injury (Yadav, et al. 2010; White and Martin, 2010). As shown previously by Sparks, et al. (1997) and Halme, et al. (2015) chlorine is released immediately and spontaneously from chloropicrin under physiological conditions. Oxidizing substances result probably from chemical reactions of chlorine (Cl\textsubscript{2}) and its hydrolysis products (e.g. HOCl, OCl\textsuperscript{-}) with cellular components (Yadav, et al. 2010). These can react further with oxygen leading to the generation of highly reactive radicals e.g. chloramines (Yadav, et al. 2010). In addition, metabolism of the subsequent de-halogenated metabolites of chloropicrin e.g. nitromethane (CH\textsubscript{3}NO\textsubscript{2}) can generate reactive nitrogen products worsening the insult. Because of the high reactivity, acute toxicity of chloropicrin damages primarily the respiratory tract where it first comes in contact with cells. Histological lesions such as ulceration, necrosis, and inflammation in the epithelium of the upper and lower parts of the respiratory tract in experimental animals have been reported after inhalation exposure to chloropicrin (EPA-report 2008).

The harmful effects of oxidizing substances on cellular proteins depend largely on the presence of metal-binding sites and the relative content of oxidation-prone amino acids (e.g. cysteine, methionine, tryptophan) in a protein (see review by Avery, 2011). Such oxidation-prone amino acids/proteins are common in the cytoskeleton. Particularly microtubules and actin filaments contain several oxidation-prone amino acids. For instance, tubulins contain 20 cysteine and 26 methionine residues (Avery, 2011; Go, et al. 2013; Gellert, et al. 2015; Roychowdhury, et al. 2000). These amino acids play an important role in folding and
polymerization of tubulins and as shown previously, their oxidation can damage filament structures (Das, et al. 2013; Gabrielsen, et al. 2013; Go, et al. 2013; Kanda, et al. 2014; Landino, et al. 2011). In this study, microscopical and immunoblotting results suggest that β-tubulin protein may be target of chloropicrin-provoked oxidation. The increased numbers of autophagy/lysosome type of vacuoles also suggests the accumulation of aggregated proteins and damaged cell organelles. Nevertheless, the up-regulation of transcripts encoding tubulin and heat shock proteins may indicate that the cells were attempting to compensate and/or to repair the damaged tubulins. Microtubule network that is composed of tubulin proteins, is involved in diverse cellular functions e.g. in cell shape and formation of the mitotic spindle during cell division. Thus, damage of β-tubulin and disruption of tubulin network will interfere with these cellular functions and lead to cell death (Janke and Bulinski, 2011; Magalhaes, et al. 2013).

The top canonical pathway enriched in this study was EIF2-signalling. This signalling is required in translation. EIF2-factor integrates diverse signals to regulate both specific and global production of proteins. Various stress conditions (e.g. unfold proteins, endoplasmic reticulum stress, oxidative stress, amino acid deprivation) modulate EIF2-signaling eliciting complex translation changes and switching activation of gene-production required for adaptation and survival in stress and reducing global protein synthesis (for a review see Holcik and Sonenberg, 2005; Ling and Söll, 2010; Shenton, et al. 2006). One probable cause for the enrichment of EIF2-signalling pathway here can be chloropicrin-evoked oxidizing compounds as seen previously after chloropicrin exposure (Pesonen et al. 2012; 2015). On the other hand, since global translation consumes much cellular energy (see Holcik and Sonnenberg, 2005), the enrichment of EIF2-signalling pathway in this study, could reflect a response to decreased energy production in mitochondria. This is suggested here by the
other enriched top pathways, mitochondrial dysfunction and increase of glycolysis. These imply that chloropicrin may have rerouted cellular energy production to the less efficient glycolysis. In particular, the up-regulation of pentose phosphate pathway that is the main pathway for the production of reducing potential (NADPH) in the cell (see review by Stincone, et al. 2015) can reflect here an attempt to restore the redox potential and that the exposure had weakened cellular redox balance in this study.

In summary, our results show that cytoskeletal β-tubulin protein is a potential target of chloropicrin exposure and this is accompanied by alterations in the transcripts related to cellular growth and proliferation and cytoskeleton in primary human bronchial epithelial cells. Furthermore, the transcript data suggest that chloropicrin may interfere with translation, protein ubiquitination and glycolysis and evoke mitochondrial dysfunction. The alterations found here in primary human epithelial cells may contribute to the development of the known pulmonary toxicity after chloropicrin exposure.

**Conflict of Interest**

The authors declare that there are no conflicts of interest.

**Supplementary material (table S1)**

The predominantly up- and down-regulated Biological Processes (BP) and KEGG-pathways after exposure of human primary bronchial epithelial cells to two concentrations (10 or 40 μM) of chloropicrin for 24 h.

**Acknowledgements**
We thank Dr Anita Naukkarinen and Dr Tarja Kokkola for valuable discussions and Pirjo Hänninen for excellent technical assistance.

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Magalhaes, H.I., Wilke, D.V., Bezerra, D.P., Cavalcanti, B.C., Rotta, R., deLima, D.P., Beatriz, A., Moraes, M.O., Diniz-Filho, J., Pessoa, C., 2013. (4-Methoxyphenyl)(3,4,5-trimethoxyphenyl) methanone inhibits tubulin polymerization induces G2/M arrest, and


Legends for the figures

Fig 1. Representative light microscopy micrographs of untreated and treated human primary bronchial epithelial cells (HBEpC). (a) Light microscopy of untreated cells and (b) cells treated with 10 μM chloropicrin or (c) 40 μM chloropicrin for 24 h, magnification 200 x.

Fig 2. Transmission electron micrographs of HBEpC-cells. (a) The cytoplasm of the untreated HBEpC-cells. (b) Deformed and aggregated (arrows) cytoskeleton structures after exposure to 10 μM or (c) to 50 μM chloropicrin for 24 h and (d) 48 h. Autophagic/lysosomal vacuoles can also be seen in the cytoplasm (arrow heads), magnification 10000x.

Fig. 3. Fluorescence micrographs of control and chloropicrin-treated HBEpC-cells stained with anti-tubulin-β antibodies. (a) Untreated, control cells, (b) the cells after exposure to 10 μM chloropicrin or (c) 40 μM chloropicrin (d) the cells without primary antibody, magnification-bar is 10 μm. Nucleus has been stained with DAPI (blue). The representative micrographs from three independent treatments performed with three replicates.

(e) Expression of β-tubulin protein in HBEpC cells after exposure to the two concentrations (10 μM and 40 μM) of chloropicrin for 24 h. Representative immunoblot (10 μg protein) and densitometric analysis of the respective columns of β-tubulin. The level of each immunodetectable protein was analysed by densitometry and normalised to loading control, β-actin. Each column represents mean ± SD from three independent experiments (C= control, CP= chloropicrin.)
Fig. 4. Top molecular networks of differentially expressed genes identified by Ingenuity Pathway Analysis (IPA) after exposure to 10 μM or 40 μM chloropicrin at 24 h in human bronchial epithelial cells (HBEpC). (a) The network of tubulins, (b) the network of actins and (c) cytokeratin. The intensity of the node colour indicates the degree of up- (red) or down-regulation (green) of the respective gene. The nodes without colour were not assessed in this study but identified by IPA as important nodes involved in these networks. Closed arrows indicate the direction of action of one gene to another, while lines without arrows indicate binding. The three replicates were used in the analysis.
Fig. 3e
Table 1. The top molecular and cellular functions, canonical pathways and upstream regulators of differentially expressed genes in primary human bronchial epithelial cells after exposure to chloropicrin for 24 h.

**A. 10 μM**

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<th>Top molecular and cellular functions</th>
<th>p-value</th>
<th>molecules</th>
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</thead>
<tbody>
<tr>
<td>Cellular growth and proliferation</td>
<td>4.36E-04 – 7.56E-22</td>
<td>150</td>
</tr>
<tr>
<td>Cellular movement</td>
<td>4.10E-04 – 3.70E-16</td>
<td>98</td>
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<tr>
<td>Cell death and survival</td>
<td>3.85E-04 – 1.68E-14</td>
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<td>Cell development</td>
<td>4.36E-04 - 2.46E-14</td>
<td>133</td>
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<tr>
<td>Protein synthesis</td>
<td>1.35E-05 – 1.90E-09</td>
<td>46</td>
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<td>EIF2 signaling</td>
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<td>Glycolysis I</td>
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<td>Oxidative phosphorylation</td>
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<tr>
<td>Glucocorticoid receptor signaling</td>
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<th>Top upstream regulators</th>
<th>p-value of overlap</th>
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<tr>
<td>TGFB1</td>
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<td>MYC</td>
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<td>5-fluorouracil</td>
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<td>D-glucose</td>
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**B. 40 μM**

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<td>Cell death and survival</td>
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<td>Protein synthesis</td>
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<tr>
<td>Cellular movement</td>
<td>5.71E-06 – 1.36E-20</td>
<td>129</td>
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<tr>
<td>Free radical scavenging</td>
<td>5.12E-06 – 2.00E-19</td>
<td>61</td>
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<td>EIF2 signaling</td>
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<td>Mitochondrial dysfunction</td>
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<td>Oxidative phosphorylation</td>
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<td>Regulation of eIF4 and p70S6K signaling</td>
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<td>mTOR signaling</td>
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<td>MYCN</td>
<td>2.91E-43</td>
</tr>
<tr>
<td>sirolimus</td>
<td>2.25E-42</td>
</tr>
<tr>
<td>CD437</td>
<td>4.04E-42</td>
</tr>
<tr>
<td>MYC</td>
<td>1.48E-36</td>
</tr>
<tr>
<td>5-fluorouracil</td>
<td>3.00E-36</td>
</tr>
</tbody>
</table>

*aOnly data with a minimum fold-change > ± 2 in mRNA was considered
Highlights

- Molecular responses of human airway epithelial cells to chloropicrin were examined
- The treatment altered cellular ultrastructure
- Chloropicrin decreased expression of cytoskeletal β-tubulin protein
- The main canonical pathways were associated with EIF2-signaling and mitochondria