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Transcriptomic Analysis of Human Primary Bronchial Epithelial Cells after Chloropicrin Treatment

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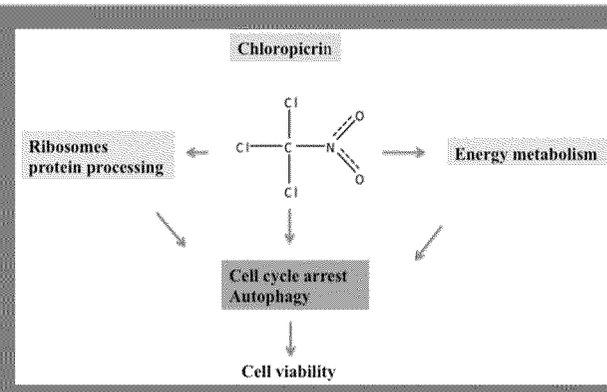
* Supporting Information

Chloropicrin is a vaporizing toxic irritant that poses a risk to human health if inhaled, but the mechanism of its toxicity in the respiratory tract is poorly understood. Here, we exposed human primary bronchial epithelial cells (HBEpC) to two concentrations of chloropicrin (10-50 μM) for 6 or 48 h and used genomic microarray, flow cytometry, and TEM-analysis to monitor cellular responses to the exposures. The overall number of differentially expressed transcripts with a fold-change > ± 2 compared to controls increased with longer exposure times. The initial response was activation of genes with a higher number of up- (512 by 10 μM and 408 by 40 μM chloropicrin) rather than down-regulated transcripts (40 by 10 μM and 215 by 40 μM chloropicrin) at 6 h seen with both exposure concentrations. The number of down-regulated transcripts, however, increased with the exposure time. The differentially regulated transcripts were further examined for enriched Gene Ontology Terms (GO) and KEGG-pathways. According to this analysis, the "ribosome" and "oxidative phosphorylation" were the KEGG-pathways predominantly affected by the exposure. The predominantly affected (GO) biological processes were "protein metabolic process" including "translation," "cellular protein complex assembly," and "response to unfolded protein." Furthermore, the top pathways, "NRF2-activated oxidative stress" and "Ah-receptor signaling," were enriched in our data sets by IPA-analysis. Real time qPCR assay of six selected genes agreed with the microarray analysis. In addition, chloropicrin exposure increased the numbers of late S and/or G2/M-phase cells as analyzed by flow cytometry and induced autophagy as revealed by electron microscopy. The targets identified are critical for vital cellular functions reflecting acute toxic responses and are potential causes for the reduced viability of epithelial cells after chloropicrin exposure.



INTRODUCTION

Chloropicrin (trichloronitromethane, CCl_3NO_2) is a colorless liquid, which volatilizes slowly and can form toxic vapors such as phosgene and nitrogen oxides when released into the environment. It is a strong irritant with the characteristics of tear gas.[1] Chloropicrin is utilized in organic syntheses and as a pesticide to fumigate soil against insects, fungi, and nematodes. Its high potency and relatively short half-life are considered as advantageous when it is used as a fumigant.[2] The irritating properties and toxicity by all routes of exposure explain why it has also been used as a chemical warfare agent.[3]

Humans are usually exposed occupationally when chloropicrin is being manufactured and handled. In addition, accidental or intentional release of chloropicrin into the environment can be another potential source of human exposure. Because of its volatility, the main route for exposure is inhalation, and exposure to chloropicrin vapor (1 ppm) provokes immediate irritation of the eyes and respiratory tract causing coughing and nausea in humans. Damage in the airways and respiratory complications have been reported to be the major injuries of chloropicrin exposure and mortality. The lethal doses for humans have been estimated to be around 120-300 ppm depending on the duration of the exposure.[1,4,5]

Analysis of chloropicrin or its metabolites in biological material has proved difficult because of its reactivity and unstable properties. Furthermore, there are no biomarkers available with which to estimate chloropicrin exposure. The only in vivo metabolic study, done in mice, has revealed that the main elimination route is through urine with a minor proportion being excreted through the lungs. In mice, di- and monocloro derivatives of chloropicrin, nitromethane, raphanusamic acid, and several unidentified polar metabolites were found in urine after intraperitoneal administration of chloropicrin.[6] The first step in chloropicrin degradation was the rapid release of chlorine and the subsequent formation of

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dechlorinated products. In addition, the mechanism of action in the respiratory system and other tissues is poorly understood. Previous studies conducted in animals and cell fractions have indicated that chloropicrin may act by inhibiting thiol-containing enzymes such as pyruvic and succinate dehydrogenases.[6,7] In addition, chloropicrin has been reported to increase the amounts of reactive oxygen species (ROS), activation of MAP-kinase (Erk1/2), and to elevate the expression of oxidative and endoplasmic reticulum stress (ER-stress) associated proteins in human cell lines.[8,9]

The development of high throughput methodologies (genomics, proteomics, and epigenomics) has made it possible to study a multitude of targets; these techniques may open new perspectives for studying mechanistic toxicology, e.g., in the development of biomarkers.[10] In particular, gene expression studies have an established place in toxicology. Nonetheless, there are well-known challenges and limitations with this kind of approach, for example, the need to utilize appropriate bioinformatics tools to process the data.[10-12] A genome-wide approach using microarray technology makes it possible to measure the expression of a large number of genes simultaneously. These kinds of data sets can provide valuable information on how chemical exposure can change gene expression and functions within the cell, and it can help to clarify the mechanisms underlying chemically induced toxicity.[13,14] Since chloropicrin is a risk to humans especially when inhaled, we used primary human bronchial epithelial cells (HBEpC) as our experimental model and applied microarray technology to study the global gene expression profile after chloropicrin exposure. Furthermore, we analyzed how the exposure affects the cell cycle and cell ultrastructure. The aim was to identify early responses, which could underlie chloropicrin toxicity in the bronchial epithelium.

MATERIALS AND METHODS

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

Chemicals. Ribonuclease A, 3-(4,5-dimethylthiazole-2-yl)-2,5-diphenyltetrazolium bromide (MTT), dimethyl sulfoxide (DMSO), propidium iodide, and Tri-Reagent were all purchased from Sigma-Aldrich (Helsinki, Finland), bronchial epithelial cell growth medium from European Collection of Cell Cultures (ECACC, Salisbury, UK), and chloropicrin (CAS#: 76-06-2) from the Defense Forces Technical Research Centre (Lakiala, Finland). The Turbo DNA-free Kit and Taqman Primer Probe Sets were from Ambion/Applied Biosystems (Waltham, USA). The 10 cm culture plates were from Sarstedt Inc. (Newton, USA) and 48-well plates from Nunc (Roskilde, Denmark).

Cell Culture and Treatment. Human primary bronchial epithelial (HBEpC (502-05a) cells were purchased from ECACC (Salisbury, UK). The cells were cryopreserved at first passage and were grown in bronchia/trachea epithelial cell growth medium at 37 °C in a humidified incubator (with 5% CO_2 and 95% air) according to the instructions provided by ECACC. In the experiments, the cells were seeded at densities that allowed the untreated cells to reach a nearly confluent state at the end of experiments. The cells were treated with increasing concentrations (1-100 μM) of chloropicrin or 0.1% DMSO as the vehicle after 48 h of plating in fresh medium and harvested at 6-48 h after the treatment. The vehicle control (0.1% DMSO) did not have any effects on the measured parameters when compared to the medium control without DMSO.

Cell Viability. The viability of the cultured cells was determined by the MTT-reduction assay described previously.[8] The cells were seeded into 48-well plates and exposed to various concentrations (1-100 μM) of chloropicrin for 24 h. After the exposure, the medium was changed to MTT-containing (0.5 mg/mL) medium and the incubation continued at 37 °C for 2 h. Thereafter, the medium was removed and the formazan crystals formed were solubilized with SDS-HCl-buffer (pH 4.7). Absorbance was measured at 570 nm with an ELx800 plate reader (Biotek Instruments Inc., Winooski, USA). The results were expressed as a percentage of the control exposed to DMSO to the same concentration used as the solvent for chloropicrin. Four independent experiments, containing 3-4 replicates at every exposure concentration were performed. The significance of the differences between exposures and respective controls was analyzed by one-way ANOVA followed by Tukey's multiple comparison test. The statistical analysis was performed using Graph Pad Prism (San Diego, USA), with $p < 0.05$ being considered as statistically significant. The concentration of chloropicrin that reduced cell viability by 50% (EC_{50}-value) was estimated by using the four-parameter logistic regression procedure according to Graph Pad Prism.

Flow Cytometric Analysis. Flow cytometric analysis after propidium iodide (PI) staining was conducted as previously described.[9] The stained cells were analyzed by FACS Canto II equipment (BD-FacsDiva, version 6.1.3, BD Biosciences, San Jose, USA).

Transmission Electron Microscopy (TEM). After exposure, the cells were fixed with 2.5% glutaraldehyde in phosphate buffer (pH 7.4) and postfixed in 1% osmium tetraoxide (OsO_4) for 3 h. The cells were then dehydrated with increasing concentrations of ethanol and embedded in Epon (LX-112). The blocks were sectioned into ultrathin slices and double stained with uranyl acetate and lead citrate. The samples were examined with a transmission electron microscope (JEM-2100F, from Jeol, Japan).

Extraction of Total RNA. In the microarray and RT-qPCR experiments, the HBEpC-cells were treated with two concentrations of chloropicrin, one low dose (10 μM) and the other dose close to the EC_{50}-value (40 μM) for either 6 or 48 h. Three replicates at each concentration and the respective controls without chloropicrin were used in exposures. After treatment, the cells were washed with PBS-solution, and the total RNA was extracted using TRI-reagent as described previously.[15] In microarray analyses, the three replicates were pooled, and the analysis was carried out using RNA-preparations from separate HBEpC-cell exposures to those used for RT-qPCR-analysis. The extracted RNA was stored at -80 °C before analysis.

Microarray Analysis of Global Transcript Levels. RNA samples were first labeled using the IlluminaR Total PrepT RNA Amplification Kit (Catalog number AMIL179, Life Technologies) according to the manufacturer's instructions, with 350 ng of RNA per sample. The quality of all cRNA samples was controlled on 2100 Bioanalyzer RNA nanochips (Agilent Technologies) and quantified using a Nanodrop 2000 spectrometer (Thermo Scientific). A total of 750 ng of each sample was used in the Direct Hybridization Assay Workflow (Illumina) using HumanHT-12 expression beadchips (Illumina). The bead-arrays were scanned using a HiScan instrument (Illumina). Raw data from the imaging were imported into the Genome Studio software using the Gene Expression Module, and RNA transcripts were quantified using the manifest file HumanHT12_V4_0_R2_15002873_B.bgx. The work was carried out at the Core Facility of the Estonian Genome Center, University of Tartu (an Illumina CSPro lab). The data were preprocessed and normalized to median with Chipster (CSC, Espoo, Finland). The differentially expressed gene sets from pooled samples were selected based on filtering by a fold-change > ±2 as compared to the respective controls. The genes representing high, moderate, and low expression levels obtained in the microarray analysis were later verified by the RT-PCR technique (see below). The lists of up- or down-regulated gene sets were analyzed by utilizing DAVID 6.7 (NIAID, NIH, Frederick, MD, USA) to extract and condense statistically enriched biological themes, with the focus on enrichment in gene ontology (GO)-terms of biological processes level 4 (GOTERM_BP_4) and KEGG-pathways.[16,17] Ingenuity pathways analysis (IPA) (Qiagen, Redwood City, CA, USA) was used to identify networks of interacting genes and top upstream regulators from the data sets of differentially expressed genes.

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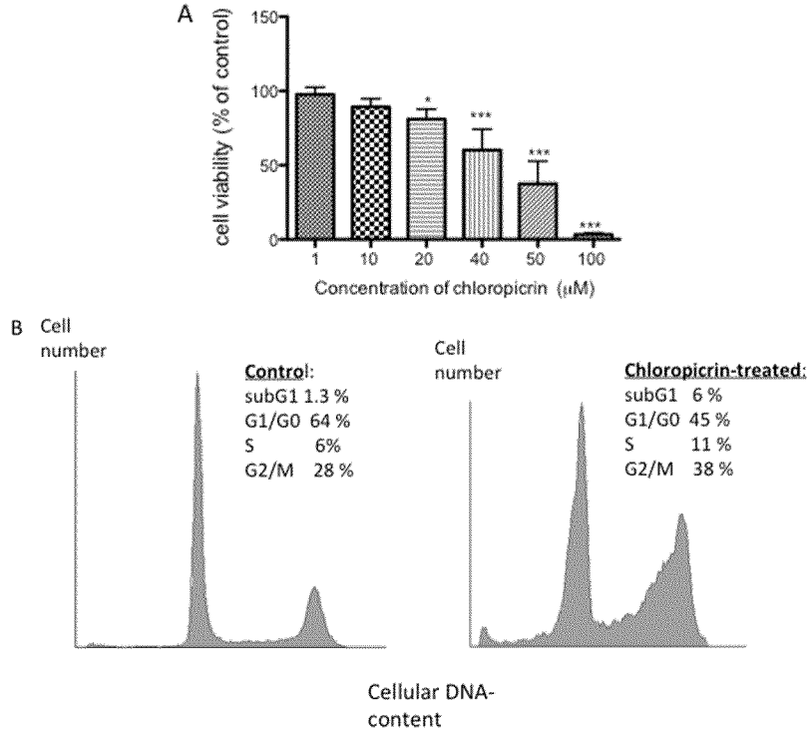

Figure 1. Effect of chloropicrin on primary human bronchial epithelial cell (HBEpC) viability. (A) Cell viability was assessed by the MTT-assay in chloropicrin-treated (24 h) HBEpC cells, expressed as a percentage of control cells. Each column represents the mean ± SD of four independent experiments, each performed with 3-4 replicates (*$p < 0.05$, ***$p < 0.01$). One-way ANOVA and Tukeys's multiple comparison tests were used. (B) Flow cytometry of propidium iodide stained HBEpC cells, the control, and those treated with chloropicrin (40 μM) for 24 h. The representative profiles and the percentages represent means from three independent experiments.

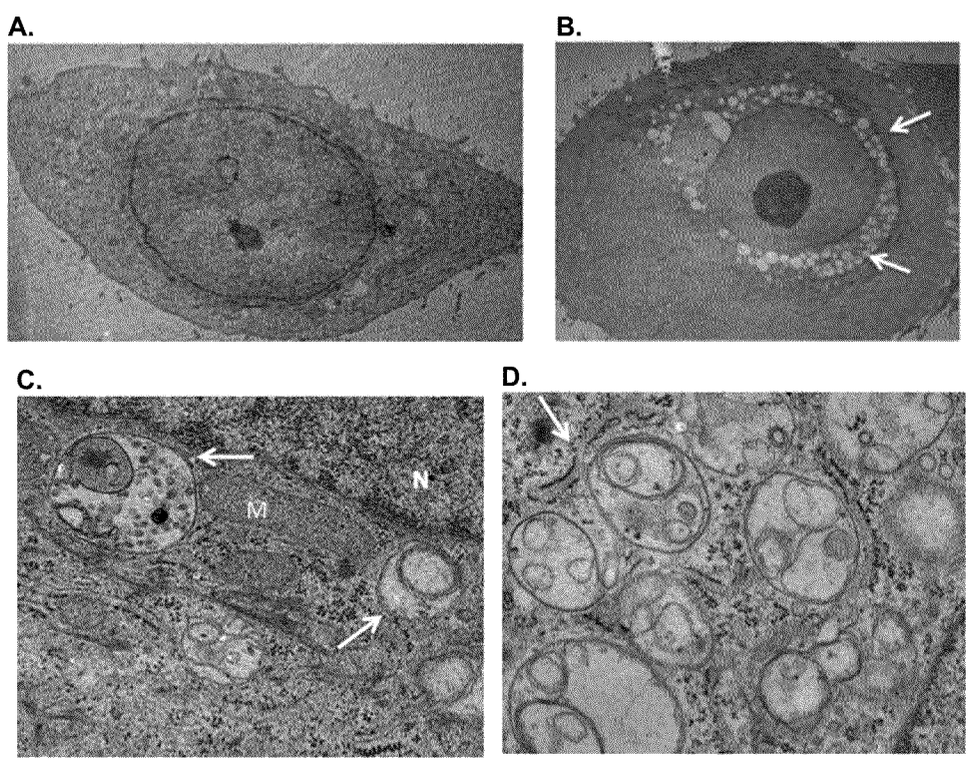

Figure 2. Representative transmission electron micrographs of HBEpC cells. (A) Untreated HBEpC cells and (B) the cells treated with 50 μM chloropicrin for 24 h. A number of vacuoles (arrows) can be seen in the perinuclear area of the treated cell (magnification 1200×). (C) Structures of typical vacuoles (arrows) seen in the HBEpC cells after the exposure to chloropicrin (50 μM) for 24 h (N nucleus, M mitochondia) and (D) for 48 h (magnification 10000×).

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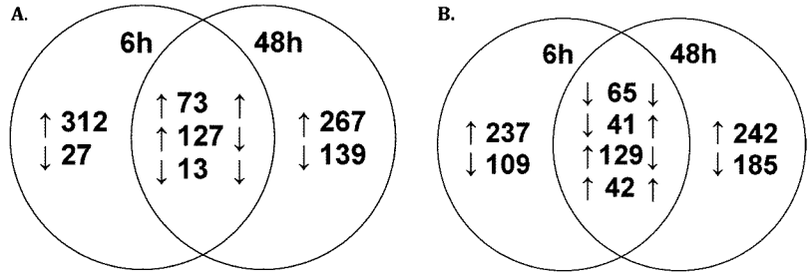

Figure 3. Venn diagrams showing the number of differentially expressed (up or down) transcripts at the two exposure time points (6 or 48 h) in primary human bronchial epithelial cells (HBEpC) after exposure to the two different concentrations of chloropicrin: (A) 10 μM or (B) 40 μM. The differentially expressed genes from the microarray study were selected based on filtering by a fold-change > ±2 in comparison to controls.

Quantitative Real-Time PCR (RT-qPCR). A RT-qPCR-study was carried out according to Storvik et al.,[15] after exposure of HBEpC-cells to chloropicrin for 6 h. Complementary DNA (cDNA) was synthesized with M-MuLReverse Transcriptase using 2 μg of total RNA in the synthesis. The RT-qPCR assay contained 40 ng of template cDNA and was carried out using an ABI Prism 7500-instrument (Applied Biosystems, USA). The amplification conditions were 50 °C for 2 min and 95 °C for 10 min followed by 40 cycles at 95 °C for 15 s and at 60 °C for 1 min. Detection was performed using six different Taqman primer probes. Two of these genes (CRYAB and HSPA6) responded highly, three (ATF3, HMOX1, and MYLIP) moderately, and one gene (ODC1) weakly to chloropicrin in the microarray assay (Table 2). Gene expression was normalized with a reference gene, β-actin, and each sample was measured in triplicate. The normalized relative mRNA expression level for each gene was expressed as a fold-change relative to the control.[18] Three replicates were used in experiments.

RESULTS

Cell Viability and Cell Cycle. To determine appropriate concentrations of chloropicrin for the subsequent microarray and other experiments, the HBEpC-cells were exposed to increasing concentrations of chloropicrin (1 - 100 μM) for 24 h, and cell viability was measured with the MTT-assay (Figure 1). Chloropicrin decreased cell viability in a dose-dependent manner. The decrease was statistically significant at chloropicrin concentrations of 20 μM or higher. The concentration that reduced cell viability by 50%, as estimated by curve fitting, was 45 μM (data not shown). Consequently, the concentrations close to this value were used in the subsequent experiments.

To study whether cytotoxicity would be associated with apoptosis or cell cycle arrest, control and chloropicrin-treated cells (10 or 40 μM for 24 h) were analyzed by flow cytometry after PI staining (Figure 1). Chloropicrin exposure (40 μM) increased slightly the number of apoptotic cells (subG1-phase), decreased the G1-phase cells, and clearly increased the late S and G2/M-phase cells in comparison with the respective control values. The lower chloropicrin concentration (10 μM) increased the number of apoptotic cells from 1.3% up to 10%, whereas the percentages of cells in the G1/G0, S, and G2/M-phases remained in the same range as those in the control cultures (data not shown).

Transmission Electron Microscopy. To get insight into the effect of chloropicrin on cellular ultrastructure, the cells were treated with one low concentration (10 μM) of chloropicrin and another somewhat higher concentration (50 μM) than that used in the microarray (40 μM) for 24 and 48 h and analyzed by transmission electron microscopy. Both concentrations increased autophagy at 24 and 48 h when compared to that of control cells. The typical finding induced by the exposure was an increase of perinuclear vacuoles, which mostly contained cytoplasmic material resembling autophagy vacuoles (Figure 2). A few of the vacuoles were empty, and these are probably zones of the swollen ER. Some mitochondria and nuclei were swollen by the higher concentration of chloropicrin (50 μM); otherwise, they stayed intact.

Genes Differentially Regulated by Chloropicrin in Human Primary Bronchial Epithelial Cells. The differentially expressed gene sets from the microarray study were selected based on the fold-change > ±2 (up or down) as compared to the untreated controls. In general, the overall number of differentially expressed transcripts after the exposure increased with an increase in the chloropicrin concentration and exposure time (i.e., from 552 to 623 at 6 h, and from 619 to 700 at 48 h) (Figure 3). The initial response was activation of genes with a higher number of up- than down-regulated transcripts at 6 h, and this trend was seen with both exposure concentrations (Figure 3). However, the number of down-regulated transcripts increased as the chloropicrin concentration and exposure time increased. There were also a large number of overlaps of differentially regulated transcripts between the 6 and 48 h of exposures (Figure 3). The complete data, showing up- and down-regulated transcripts at the two time points, are shown as Supporting Information (Table S1). Since the microarray data are semiquantitative, the differentially regulated genes were subjected to an enrichment analysis in an attempt to reveal statistically significant themes as designated in the GO Terms and KEGG-pathways. According to these analyses, the ribosome pathway and related protein processing, energy metabolism, and regulation of cell death were ranked as the main KEGG-pathways and biological processes being affected by the chloropicrin treatment (Table 1).

Possible Effect on Protein Processing. The "ribosome-pathway" was one of the most enriched KEGG-pathways by chloropicrin exposure both at 6 and 48 h (Table 1). The up-regulated pathway consisted of transcripts encoding many eukaryotic translation initiation factors (e.g., EIF2A, EIF1B, and EIF3K) and structural constituents of ribosome and RNA-binding proteins (e.g., RPL13A, RPL14, and RPL15). These are consistent with the transcripts associated with the GO-term "protein metabolic process" that includes "translation" (Table S2). In particular, they contained the transcripts encoding for proteins, which are important for protein synthesis and degradation (Table S2). At 6 h, the enriched GO-term "response to unfolded protein" contained transcripts that are inducible in conditions of endoplasmic reticulum stress (e.g., DDIT3, HERPUD1, DNAJA1, and PPP1R15A) suggesting alterations in ER-functions (Tables 1A and S2). The "ribosome pathway" and the "translation" were still up-regulated at 48 h with the higher exposure concentration, although the number

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Table 1A. Predominantly Enriched (GO) Biological Processes (BP) and KEGG-Pathways in Human Primary Bronchial Epithelial Cells after Exposure to Chloropicrin Upregulated at 6 h

	10 μM		
GOTERM_BP_4	n	p-value	benjamini
translation	50	4.3×10^{-30}	3.5×10^{-27}
oxidative phosphorylation	20	2.2×10^{-14}	8.9×10^{-12}
electron transport chain	18	4.5×10^{-11}	1.2×10^{-8}
cellular protein metabolic process	85	1.2×10^{-9}	2.5×10^{-7}
respiratory electron transport chain	13	2.3×10^{-9}	3.7×10^{-7}
protein metabolic process	92	2.6×10^{-8}	3.5×10^{-6}
energy derivation by oxidation of organic compounds	15	6.0×10^{-7}	6.9×10^{-5}
proton transport	10	2.0×10^{-6}	2.0×10^{-4}
	10 μM		
KEGG_PATHWAY	n	p-value	benjamini
oxidative phosphorylation	33	8.5×10^{-22}	8.4×10^{-20}
Parkinson's disease	28	1.3×10^{-16}	5.4×10^{-15}
ribosome	24	1.4×10^{-16}	3.7×10^{-15}
Huntington's disease	29	1.4×10^{-13}	3.5×10^{-12}
Alzheimer's disease	26	4.6×10^{-12}	9.2×10^{-11}
cardiac muscle contraction	10	4.3×10^{-4}	7.0×10^{-3}
	40 μM		
GOTERM_BP_4	n	p-value	benjamini
translation	39	7.2×10^{-21}	6.2×10^{-18}
response to unfolded protein	17	5.3×10^{-14}	2.3×10^{-11}
cellular protein metabolic process	86	2.1×10^{-12}	6.0×10^{-10}
protein metabolic process	91	2.9×10^{-10}	6.2×10^{-8}
regulation of programmed cell death	40	5.2×10^{-9}	9.0×10^{-7}
regulation of cell death	40	5.7×10^{-9}	8.3×10^{-7}
negative regulation of apoptosis	23	2.2×10^{-7}	2.7×10^{-5}
	40 μM		
KEGG_PATHWAY	n	p-value	benjamini
ribosome	19	1.5×10^{-12}	1.4×10^{-10}
oxidative phosphorylation	16	4.8×10^{-7}	2.4×10^{-5}
Parkinson's disease	15	2.3×10^{-6}	7.6×10^{-5}
Huntington's disease	16	2.9×10^{-5}	7.1×10^{-4}
Alzheimer's disease	12	2.1×10^{-3}	4.0×10^{-2}
aminoacyl-tRNA biosynthesis	6	3.1×10^{-3}	4.9×10^{-2}

of altered transcripts was lower than that at 6 h (Tables 1B and S2).

There were an elevated number of the down-regulated transcripts in the "ribosome pathway" (e.g., RPS2, RPL3, RPLP0, and RPLP2) in response to the increase in the chloropicrin concentration and the exposure time (Tables 1C and 1D). Furthermore, at 48 h, the GO-terms "cellular amino acid metabolic process" consisted of down-regulated transcripts encoding transcription factor 4 (ATF4) and various amino acid tRNA-synthetases (e.g., AARS, MARS, and NARS).

Possible Effects on Energy Metabolism. The other KEGG-pathway predominantly enriched at 6 and 48 h (Tables 1A and 1B) was "oxidative phosphorylation", which included the transcripts encoding for components of the mitochondrial respiratory chain, in particular the components of complexes I, IV, and V (Table S2). The other energy metabolism pathway enriched at 48 h was "glycolysis/gluconeogenesis" (Tables 1B and S2) suggesting that chloropicrin enhanced the cells' need for energy resources.

Table 1B. (B) Predominantly Enriched (GO) Biological Processes and KEGG-Pathways in Human Primary Bronchial Epithelial Cells after Exposure to Chloropicrin Upregulated at 48 h

	10 μM		
GOTERM_BP_4	n	p-value	benjamini
regulation of cell death	34	5.2×10^{-7}	4.9×10^{-4}
cellular macromolecular complex assembly	20	6.9×10^{-7}	3.2×10^{-4}
regulation of programmed cell death	33	1.4×10^{-6}	4.5×10^{-4}
cellular protein complex assembly	14	1.7×10^{-6}	4.0×10^{-4}
glycolysis	8	8.1×10^{-6}	1.5×10^{-3}
	10 μM		
KEGG_PATHWAY	n	p-value	benjamini
pathogenic Escherichia coli infection	11	8.5×10^{-7}	9.3×10^{-5}
glycolysis/gluconeogenesis	8	5.7×10^{-4}	3.1×10^{-2}
	40 μM		
GOTERM_BP_4	n	p-value	benjamini
translation	19	3.3×10^{-10}	1.7×10^{-7}
oxidative phosphorylation	9	1.8×10^{-6}	4.4×10^{-4}
respiratory electron transport chain	6	2.1×10^{-4}	3.4×10^{-2}
negative regulation of ubiquitin-protein ligase activity during mitotic cell	6	2.2×10^{-4}	2.8×10^{-2}
positive regulation of ubiquitin-protein ligase activity during mitotic cell	6	2.8×10^{-4}	2.7×10^{-2}
regulation of ubiquitin-protein ligase activity during mitotic cell cycle	6	3.4×10^{-4}	2.8×10^{-2}
protein complex assembly	14	3.5×10^{-4}	2.5×10^{-2}
	40 μM		
KEGG_PATHWAY	n	p-value	benjamini
ribosome	14	3.3×10^{-10}	1.7×10^{-7}
pathogenic Escherichia coli infection	9	1.7×10^{-6}	4.4×10^{-4}
oxidative phosphorylation	11	2.2×10^{-5}	3.4×10^{-2}
gap junction	7	2.1×10^{-3}	2.8×10^{-2}
Alzheimer's disease	9	3.0×10^{-3}	2.7×10^{-2}
Parkinson's disease	8	3.0×10^{-3}	2.8×10^{-2}

Table 1C. Predominanatly Enriched (GO) Biological Processes and KEGG-Pathways in Human Primary Bronchial Epithelial Cells after Exposure to Chloropicrin Down-regulated at 6 h

	10 μM		
GOTERM_BP_4	n	p-value	benjamini
translation	9	6.6×10^{-11}	2.5×10^{-9}
cellular protein metabolic process	10	2.4×10^{-5}	4.6×10^{-4}
protein metabolic process	10	1.1×10^{-4}	1.3×10^{-3}
cellular macromolecule biosynthetic process	9	9.2×10^{-4}	8.7×10^{-3}
	10 μM		
KEGG_PATHWAY	n	p-value	benjamini
ribosome	9	5.3×10^{-15}	5.3×10^{-15}
	40 μM		
GOTERM_BP_4	n	p-value	benjamini
translation	20	2.3×10^{-9}	1.8×10^{-6}
RNA processing	18	7.5×10^{-5}	2.8×10^{-2}
	40 μM		
KEGG_PATHWAY	n	p-value	benjamini
ribosome	12	5.1×10^{-8}	5.6×10^{-6}
focal adhesion	11	8.1×10^{-4}	4.3×10^{-2}

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Table 1D. Predominantly Enriched (GO) Biological Processes (BP)and KEGG-Pathways in Human Primary Bronchial Epithelial Cells after Exposure to Chloropicrin Down-regulated at 48 h

	10 μM		
GOTERM_BP_4	n	p-value	benjamini
translation	35	1.8×10^{-25}	1.1×10^{-22}
cellular protein metabolic process	43	5.0×10^{-7}	1.5×10^{-4}
regulation of programmed cell death	25	1.1×10^{-6}	2.3×10^{-4}
regulation of cell death	25	1.2×10^{-6}	1.9×10^{-4}
protein metabolic process	51	6.0×10^{-6}	2.3×10^{-4}
cellular amino acid metabolic process	13	2.4×10^{-6}	2.4×10^{-4}
cellular amine metabolic process	15	1.8×10^{-6}	5.3×10^{-4}
	10 μM		
KEGG_PATHWAY	n	p-value	benjamini
ribosome	20	7.3×10^{-17}	8.7×10^{-15}
oxidative phosphorylation	12	9.6×10^{-6}	3.7×10^{-4}
	40 μM		
GOTERM_BP_4	n	p-value	benjamini
translation	44	4.0×10^{-25}	4.0×10^{-22}
cellular protein metabolic process	74	6.2×10^{-7}	3.1×10^{-4}
protein metabolic process	83	1.2×10^{-6}	3.9×10^{-4}
negative regulation of cellular process	55	7.5×10^{-6}	1.9×10^{-3}
regulation of programmed cell death	34	8.3×10^{-6}	1.6×10^{-3}
regulation of cell death	34	8.9×10^{-6}	1.5×10^{-3}
	40 μM		
KEGG_PATHWAY	n	p-value	benjamini
ribosome	30	3.4×10^{-24}	4.1×10^{-22}
Parkinson's disease	17	6.1×10^{-7}	3.7×10^{-5}
Alzheimer's disease	18	3.5×10^{-6}	1.4×10^{-4}
oxidative phosphorylation	16	3.9×10^{-6}	1.2×10^{-4}

Possible Effect on the Regulation of Cell Death. A large number of the up-regulated transcripts were also associated with the GO-term, "regulation of cell death" both at 6 and 48 h (Tables 1 and S2). These consisted of apoptotic and antiapoptotic as well as oxidative stress and calcium responding and tubulin encoding transcripts (e.g., CRYAB, HMOX1, CALR, TUBB, DDIT3, STK3, and SOD1). At 48 h, there were a number of down-regulated transcripts, e.g., those encoding for, e.g., thiol-protease, amino acid tRNA-synthetases, immunological responses, and cell cycle arresting proteins (e.g., CTSB, ASNS, IL1A, and CDKN1A) (Tables 1D and S2).

Verification of Microarray Data by RT-qPCR. Quantitative real-time RT-qPCR was used to verify the six genes (ATF3, CRYAB, HMOX1, HSPA6, MYLIP, and ODC1) that responded to chloropicrin at the 6 h exposure time point in the microarray study (Table 2 and S1). In general, the methods showed good agreement between the mRNA levels, and with the lower dose (10 μM), the levels were almost identical. With the higher dose (40 μM), which increased the dying cells, the quantitative differences in fold-changes were more notable (Table 2).

Analysis of Pathways and Upstream Regulators. To identify pathways that are most significant to microarray data, the differentially expressed genes at 6 h were mapped into functional networks by performing an IPA analysis. As shown in Figure 4, the main network was cancer, organismal injury and abnormalities, and reproductive system disease (Figure 4A). Figure 4B shows also the top upstream regulators and their target molecules identified from our data sets by the IPA tool.

Table 2. Comparison of Microarray Results with the RT-qPCR[a] after Exposure of HBEpC-Cells to Chloropicrin for 6 h

			fold-change	
gene	description	chloropicrin (μM)	microarray[b]	QRT-PCR[c]
ATF3	activating transcription	10	1.3	2.6
	factor 3	40	6.8	37.9
CRYAB	alpha-basic crystalline	10	2.8	4.0
		40	40.7	44.7
HMOX1	hemioxygenase-1	10	3.9	2.9
		40	4.4	4.7
HSPA6	heat shock A6	10	4.8	1.5
		40	58.8	8.3
MYLIP	myosin regulatory	10	1.2	1.2
	light chain	40	7.5	35.2
ODC1	ornithine decarboxylase	10	1.0	1.0
		40	1.1	1.4

[a]RT-qPCR was performed on cDNAs for the indicated transcripts, compared to the expression of the housekeeping β-actin gene, and normalized to the solvent control. [b]Data are the mean from a pooled sample from three replicate exposures. [c]Data represent the mean of the three replicate exposures (independent from microarray exposures).

The TP63-gene (encodes for tumor protein 63) is involved in DNA-damage, cell cycle control, and apoptosis through p53 and calcium signaling. The second main enriched gene was the ZEB2-gene (encodes for the zinc finger transcription regulator); this is associated with the epithelial - mesenchymal transition pathway and is expressed in late S/G2/M phase. The target molecules of the five upstream-regulators listed in Figure 4B are associated with cell adhesion, protein folding, repair of damaged DNA, regulation of cell cycle, synthesis of fatty acids, and the proteins required for movement and stabilization of microfilaments. In addition, according to the IPA-analysis, "(NRF2)-activated oxidative stress" (p 8.6×10^{-04}) and "AhR-signalling" (p 2.0×10^{-03}) were the top pathways enriched by the treatment.

DISCUSSION

Chloropicrin is an evaporating toxic irritant that is a human health risk if inhaled.[1] Utilizing genome-wide mRNA-expression and analysis of KEGG-pathways and biological processes, we show that chloropicrin alters several transcripts and pathways in human primary bronchial epithelial cells. The microarray data and the six transcripts analyzed with RT-qPCR from a replicated experiment were in agreement suggesting that the microarray data, although semiquantitative, was able to capture the relevant alterations evoked by chloropicrin. In order to reduce the effect of potential arbitrary individual transcripts in the interpretation, a thematic analysis was performed, and the list of genes was condensed into enriched pathways. The majority of the genes altered by the exposure were associated with protein processing (synthesis and metabolism), energy metabolism, and regulation of cell cycle and cell death.

The predominantly affected transcripts were associated with protein processing (ribosomes). In stress conditions, protein synthesis and metabolism are multifaceted and closely related to many processes in the ER.[19,20] In this study, there were several initially highly expressed transcripts, e.g., the heat shock (chaperone) transcripts (e.g., HSPA1A, DNAJB4, HSPA6, and

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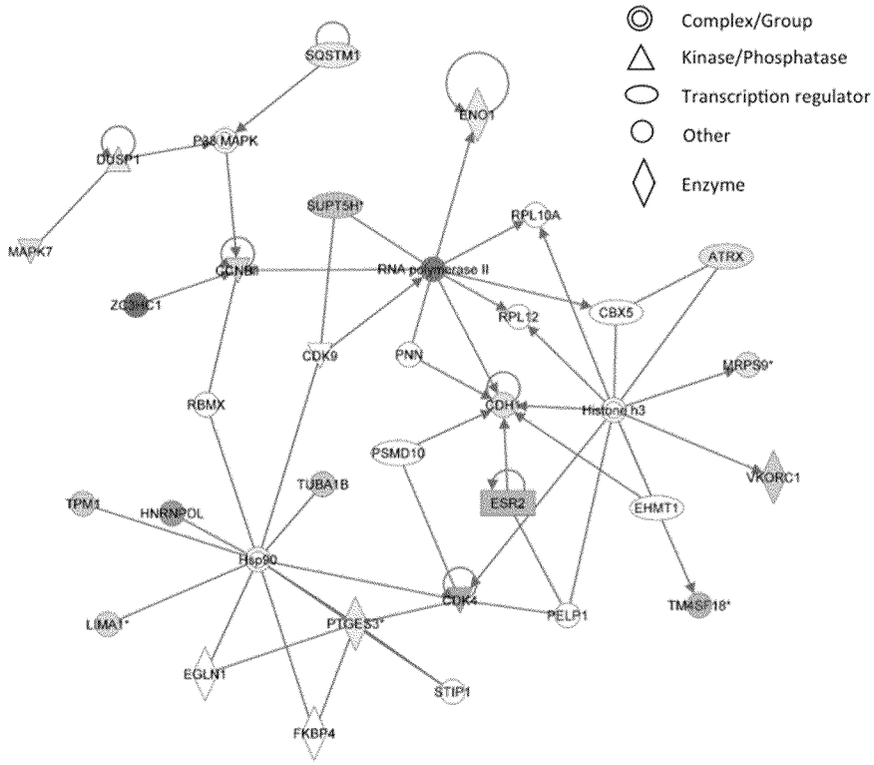

A

Molecules in Network	Score	Focus Molecules	Top Functions
ATRX, CBX5, **CCNB1**, **CDH1**, **CDK4**, CDK9, **DUSP1**, EGLN1, EHMT1, **ENO1**, **ESR2**, FKBP4, Histone h3, **HNRNPDL**, Hsp90, **LIMA1**, **MAPK7**, **MRPS9**, P38 MAPK, PELP1, PNN, PSMD10, **PTGES3**, RBMX, RNA polymerase II, RPL12, RPL10A, **SQSTM1**, STIP1, **SUPT5H**, **TM4SF18**, **TPM1**, **TUBA1B**, **VKORC1**, **ZC3HC1**	25	19	Cancer, Organismal Injury and Abnormalities, Reproductive System Disease

B

Top upstream regulators	Target molecules
TP63	FASN, FBN1, HBP1, IGFBP6, KIF23, TPM1
ZEB2	CDH1, KRT19
GPER1	ASAH1, FASN
E2F1	CCNB1, CDK4, CWC27, HLTF, KIF23
SMARCA4	A2M, HBG1, LGALS1, SMARCA2, TPM1

Figure 4. Top molecular network of differentially expressed genes after exposure to 10 μM or 40 μM of choropicrin at 6 h in human bronchial epithelial cells (HBEpC) (A) IPA identified the most significant molecular network depicting genes involved in cancer, organismal injury and abnormalities, and reproductive system disease. The intensity of the node color indicates the degree of up- (red) or down-regulation (green) of the respective gene. The nodes without color were not assessed in this study but identified by IPA as important nodes involved in the network. Closed arrows indicate the direction of action of one gene to another, while lines without arrows indicate binding. A key to the identity of node shapes is included in the figure. Molecules shown in bold in Table 1A are those identified in Table S1. Table 1B shows top upstream regulators and their target molecules of differentially expressed genes in HBEpC after exposure to chloropicrin (10 μM or 40 μM) for 6 h analyzed by the IPA tool.

HSP9OAA1), which are known to restore unfolded/misfolded proteins, prevent aggregation, and facilitate selective degradation of proteins.[21-24] Furthermore, a large number of altered transcripts required in translation and synthesis of ribosomal proteins suggest that the exposure interferes with protein synthesis. In addition, increased expression of the transcripts (TRIB3, MYLIP, and HERPUD1) involved in protein degradation through the ubiquitin-proteosome-pathway hints at an accumulation of misfolded proteins in the endoplasmic reticulum. By switching on the transcription of the genes related to the chaperones and protein degradation, the cells are attempting to increase their capability to restore or remove the misfolded proteins generated by the exposure. The altered ER-functions are the most likely cause for increased autophagy seen by the electron microscopy in this study. Autophagy that has a critical role in removing aggregated proteins and damaged organelles[25] may be activated here due to the increasing amount of degradation products in the cell. Thus, the material inside the autophagy vacuoles in this study represents probably misfolded/damaged proteins and other cytoplasmic structures.

Protein processing in the ER is highly sensitive to alterations in the redox balance of the cell,[26] and this is a possible early cause for the changes seen here in the transcript levels. The evidence for this proposal emerges from the enriched top pathway, "NRF2-regulated oxidative stress." NRF2 is a redox sensing transcription factor activated by electrophilic and oxidative stress. It regulates a number of genes[27,28] encoding detoxification and antioxidant enzymes, e.g., HMOX1, GLRX,

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TXNRD1, and NQO1 genes, which were up-regulated in this study. NRF2 also coordinates the functions of many of the other genes that are involved in, e.g., for repair and degradation of damaged macromolecules and modulate intermediary metabolism[28] (e.g., CBR1, SQSTM1, CYP2B2, and DNAJA2 genes in this study). The other enriched top pathway, "Ah-receptor signaling," is closely related to oxidative stress and partly overlaps with the NRF2-activated pathway. The Ah-receptor is a ligand activated transcription factor that directly regulates the expression of many xenobiotic detoxification enzymes (e.g., ALDH3A, CYP1B1, and GSTA5 genes in this study), facilitating the metabolism and elimination of xenobiotics.[29] In addition, Ah-receptor signaling has been associated with a wide range of other genes[30] and pathways, e.g., energy metabolism, degradation through the ubiquitin ligase complex, and signaling via steroid receptors (e.g., FASN and ESR2 genes in this study). Disruption of the redox-balance was also supported by our previous cell culture studies, which revealed a rapid depletion of thiol-containing antioxidant glutathione as well as elevation of ROS-production and the formation of disulfide-bonds between sulfhydryl-containing amino groups (cysteine) by chloropicrin exposure.[8,9]

The acute source of the oxidative insult by chloropicrin originates probably from the reactivity of chlorine that is promptly released from chloropicrin in biological fluids.[7] The released chlorine can generate a variety of reactive intermediates in physiological fluids and thereby indirectly affect cellular redox-balance. In addition to chlorine reactivity, the metabolism of nitromethane, the dehalogenated metabolite of chloropicrin, may be another cause of the acute changes found in the transcripts in this study. In particular, the nitro group in the nitromethane molecule (CH_3NO_2) can actively take part in generating oxidative effects. The formation of nitrites, the inhibition of liver enzymes (cytochrome P450, aldose-reductase, NADPH-cytochrome reductase), and the proliferation of the endoplasmic reticulum have been reported after exposure of rodents to nitromethane[31-33] suggesting that the ER is the target of nitromethane insult.

The other predominantly up-regulated pathway enriched at both of the exposure times was oxidative phosphorylation. Furthermore, the increase of "glycolysis/gluconeogenesis" at 48 h after the exposure suggests that chloropicrin lowered the ATP/ADP-ratio favoring enhanced glycolysis. The changes in these transcripts may be a compensation response to the effects of chloropicrin on the energy metabolizing enzymes such as the pyruvate and succinate complexes or other thiol/hemicontaining enzymes as shown previously.[6,7] However, the increases in the transcripts involved in energy metabolism here may also reflect a secondary response to general stress and toxicity caused by the treatment.

Many of the altered transcripts in this study were associated with regulation of cell death consisting of both pro-apoptotic and antiapoptotic transcripts (e.g., DDIT3, DEDD2, BAG3, and DAD1). In addition, the transcripts (GADD34, GADD45B, and CDKN1A), which are associated with DNA-damage and cell cycle arrest[34-36] were clearly up-regulated by the exposure. Several of the main upstream regulators (e.g., TP63, E2F1, and SMARCA4) identified from our data sets have similar functions. Furthermore, the flow cytometry analysis confirmed that the exposure caused an increase in the number of the late S and/or G2/M-phase cells by the exposure. Since the cell cycle checkpoints are important for the maintenance of genomic stability in response to DNA damage, these results indicate that chloropicrin treatment evokes DNA damage, which then leads to late S- and/or G2/M-arrest and shifts a portion of the cells into death. This is in line with our previous studies[8,9] with chloropicrin in human epithelial cell lines, where there were increases in the amounts of several proteins (p21, p27, and p53) known to be involved in the cell cycle control after DNA-damage.[37,38] However, the G2/M phase arrest could also result from alterations of the microtubule network required for the cytokinesis and separation of chromosomes.[39-41] Support for this mechanism was found here in the increases in the transcripts for tubulin proteins, which are the principal components of microtubules.

In conclusion, these results describe mRNA changes in human primary bronchial epithelial cells. The model is very relevant because bronchial epithelial cells are part of the first line of defense against inhaled reactive chemicals after in vivo exposures. Our results indicate that critical cellular functions (protein homeostasis, energy metabolism, and regulation of cell cycle and cell death) are targets of the chloropicrin exposure. Furthermore, the top pathways, "NRF2-activated oxidative stress" and "Ah-receptor signaling" enriched in our data sets are toxicologically important and should be further evaluated at the protein level. These pathways may underlie the reduced viability of epithelial cells after chloropicrin exposure.

ASSOCIATED CONTENT

* Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.chemrestox.5b00123.

Up- or down-regulated transcripts (fold-change compared to the respective controls) after exposure of human primary bronchial epithelial cells to the two concentrations of chloropicrin (10 or 40 μM) for 6 or 48 h (XLS)

predominantly up-regulated Biological Processes (BP) and KEGG-pathways and the lists of genes (official symbol) in each of the enriched categories in human primary bronchial epithelial cells after exposure to two different concentrations of chloropicrin (10 or 40 μM) for 6 h (A) or 48 h (B); the corresponding down-regulated GO-Terms (BP) and KEGG-pathways at 6 h (C) and at 48 h (D)(XLSX)

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ABBREVIATIONS

AhR, aryl hydrocarbon receptor; DMSO, dimethyl sulfoxide; ECACC, European Collection of Cell Cultures; ER,

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endoplasmic reticulum; GO TERM, gene ontology term; BP, biological processes; HBEpC, human bronchial epithelial cells; IPA, ingenuity pathway analysis; KEGG, Kyoto Encyclopedia of Genes and Genomes; MAP-kinase, mitogen-activated protein kinase; MTT, 3-(4,5-dimethylthiazole-2-yl)-2,5-diphenyltetrazolium bromide; NRF2, nuclear factor erythroid-2-like factor; ppm, parts per million; PI, propidium iodide; ROS, reactive oxygen species; RT-qPCR, real time quantitative polymerase chain reaction; TEM, transmission electron microscopy; TRI-reagent, RNA isolation reagent

REFERENCES

(1) EPA-report (2008) Acute Exposure Guideline Levels, Chloropicrin, pp 1-52. http://www.epa.gov/oppt/aegl/pubs/chemlist.htm.

(2) Ruzo, L. O. (2006) Physical, chemical and environmental properties of selected chemical alternatives for the pre-plant use of methyl bromide as soil fumigant. Pest Manage. Sci. 62, 99-113.

(3) Sutherland, R. G. (2004) Chemical and biochemical non-lethal weapons. SPRI Policy Paper 23, 1-14.

(4) Gonmori, K., Muto, H., Yamamoto, T., and Takahashi, K. (1987) A case of homicidal intoxication by chloropicrin. Am. J. Forensic Med. Pathol. 8, 135-138.

(5) Oriel, M., Edmiston, S., Beauvais, S., Barry, T., and O'Malley, M. (2009) Illnesses Associated with Chloropicrin Use in California Agriculture, 1992-2003. In Reviews of Environmental Contamination and Toxicology (Whitacre, D. M., Ed.) pp. 1-30, Springer Science, LLC, New York.

(6) Sparks, S. E., Quistad, G. B., and Casada, J. E. (1997) Chloropicrin: Reactions with biological thiols and metabolism in mice. Chem. Res. Toxicol. 10, 1001-1007.

(7) Sparks, S. E., Quistad, G. B., Li, W., and Casida, J. E. (2000) Chloropicrin dechlorination in relation to toxic action. J. Biochem. Mol. Toxicol. 14, 26-32.

(8) Pesonen, M., Pasanen, M., Loikkanen, J., Naukkarinen, A., Hemmilä, M., Seulanto, H., Kuitunen, T., and Vähäkangas, K. (2012) Chloropicrin induces endoplasmic reticulum stress in human retinal pigment epithelial cells. Toxicol. Lett. 211, 239-245.

(9) Pesonen, M., Hakkinen, M., Rilla, K., Juvonen, R., Kuitunen, T., Pasanen, M., and Vähäkangas, K. (2014) Chloropicrin-induced toxic responses in human lung epithelial cells. Toxicol. Lett. 226, 236-244.

(10) Fowler, B. A. (2012) Biomarkers in Toxicology and Risk Assessment. In Molecular, Clinical and Environmental Toxicology (Luch, A., Ed.) pp 459-470, Springer Basel AG, Basel, Switzerland.

(11) Fielden, M. R., and Zacharewski, T. R. (2001) Challenges and limitations of gene expression profiling in mechanistic and predictive toxicology. Toxicol. Sci. 60, 6-10.

(12) Jennings, P., Limonciel, A., Feliece, L., and Lenonard, M. O. (2013) An overview of transcriptional regulation in response to toxicological insult. Arch. Toxicol. 87, 49-72.

(13) Chen, M., Zhang, M., Borlak, J., and Tong, W. (2012) A decade of toxicogenomic research and its contribution to toxicological science. Toxicol. Sci. 130, 217-228.

(14) McHale, C. M., Zhang, L., Thomas, R., and Smith, M. T. (2013) Analysis of the transcriptome in molecular epidemiology studies. Environ. Mol. Mutagen. 54, 500-517.

(15) Storvik, M., Huuskonen, P., Kyllönen, T., Lehtonen, S., El-Nezami, H., Auriola, S., and Pasanen, M. (2011) Aflatoxin B1- a potential endocrine disruptor-up-regulates CYP19A1 in JEG-3 cells. Toxicol. Lett. 202, 161-167.

(16) Huang, D. W., Sherman, B. T., and Lempicki, R. A. (2008) Systematic and integrative analysis of large gene lists using DAVID Bioinformatics Resources. Nat. Protoc. 4, 44-57.

(17) Huang, D. W., Sherman, B. T., and Lempicki, R. A. (2009) Bioinformatics enrichment tools: paths toward the comprehensive functional analysis of large gene lists. Nucleic Acids Res. 37, 1-13.

(18) Pfaffl, M. W. (2001) A new mathematical model for relative quantification in real-time RT-PCR. Nucleic Acids Res. 29, e45.

(19) Holcik, M., and Sonenberg, N. (2005) Translational control in stress and apoptosis. Nat. Rev. Mol. Cell Biol. 6, 318-327.

(20) Cao, S. S., and Kaufman, R. J. (2012) Unfolded protein response. Curr. Biol. 22, R622-R626.

(21) Qiu, X.-B., Shao, Y.-M., Miao, S., and Wang, L. (2006) The diversity of the DNAJ/HSP40 family, the crucial partners for Hsp 70 chaperones. Cell. Mol. Life Sci. 63, 2560-2570.

(22) Pratt, W. B., Morishima, Y., Peng, H.-M., and Osawa, Y. (2010) Proposal for a role of the Hsp90/Hsp70-based chaperone machinery in making triage decisions when proteins undergo oxidative and toxic damage. Exp. Biol. Med. 235, 278-289.

(23) Hageman, J., van Waarde, M. A., Zylicz, A., Walerych, D., and Kampinga, H. H. (2011) The diverse members of the mammalian HSP70 machine show distinct chaperone-like activities. Biochem. J. 435, 127-142.

(24) Wek, R. C., and Cavener, D. R. (2007) Translational control and the unfolded protein response. Antioxid. Redox Signaling 9, 2357-2371.

(25) He, C., and Klionsky, D. J. (2009) Regulation mechanisms and signaling pathways of autophagy. Annu. Rev. Genet. 43, 67-93.

(26) Malhotra, J. D., and Kaufman, R. J. (2007) Endoplasmic reticulum stress and oxidative stress: A vicious cycle or a double-edged sword? Antioxid. Redox Signaling 9, 2277-2293.

(27) Zenkov, N. K., Menshchikova, E. B., and Tkachev, V. O. (2013) Keap1/NRF2/ARE redox-sensitive signaling system as a pharmacological target. Biochemistry 78, 19-36.

(28) Hayes, J. D., and Dinkova-Kostova, A. T. (2014) The Nrf2 regulatory network provides an interface between redox and intermediary metabolism. Trends Biochem. Sci. 39, 199-217, doi.org/10.1016/j.tibs.2014.02002,.

(29) Safe, S., Lee, S.-O., and Jin, U.-H. (2013) Role of the Aryl Hydrocarbon Receptor in Carcinogenesis and potential as a drug target. Toxicol. Sci. 135, 1-16.

(30) Ohtake, F., Baba, A., Takada, I., Okada, M., Iwasaki, K., Miki, H., Takahashi, S., Kouzumenko, A., Nohara, K., Chiba, T., Fujii-Kuriyama, Y., and Kato, S. (2007) Dioxin receptor is a ligand-dependent E3 ubiquitin ligase. Nature 446, 562-566.

(31) Sakurai, H., Hermann, G., Ruf, H. H., and Ullrich, V. (1980) The interaction of aliphatic nitro compounds with the liver microsomal monooxygenase system. Biochem. Pharmacol. 29, 341-345.

(32) IARC (2000) Monographs on Evaluation of Carcinogenic Risk to Humans: Nitromethane, Vol. 77, pp 487-501, IARC, Lyon, France.

(33) Zitting, A., Nickels, J., and Savolainen, H. (1982) Comparison of acute toxic effects of intraperitoneally injected nitromethane and nitroethane in rats. Toxicol. Lett. 13, 195-201.

(34) Dutto, I., Tillhon, M., Cazzalini, O., Stivala, L. A., and Prosperi, E. (2015) Biology of the cell cycle inhibitor $p21^{CDKN1A}$: molecular mechanisms and relevance in chemical toxicology. Arch. Toxicol. 89, 155-178.

(35) Bunz, F., Dutriaux, A., Lengauer, C., Waldman, T., Zhou, S., Brown, J. P., Sedivy, J. M., Kinzler, K. W., and Vogelstein, B. (1998) Requirement for p53 and p21 to sustain G2 arrest after DNA-damage. Science 282, 1497-1501.

(36) Vairapandi, M., Balliet, A. G., Hoffman, B., and Liebermann, D. A. (2002) GADD45b and GADD45g are cdc2/CyclinB1 kinase inhibitors with a role in S and G2/M cell cycle checkpoints induced by genotoxic stress. J. Cell. Physiol. 192, 327-338.

(37) Hussain, S. P., and Harris, C. C. (2006) p53 biological network: at the crossroad of cellular-stress response pathway and molecular carcinogenesis. J. Nippon Med. Sch. 73, 54-64.

(38) Lavin, M. F., and Gueven, N. (2006) Complexity of p53 stabilization and activation. Cell Death Differ. 13, 941-950.

(39) Lee, C.-F., Liu, C.-Y., Hsieh, R.-H., and Wei, Y.-H. (2005) Oxidative stress-induced de-polymerization of microtubules and alteration of mitochondrial mass in human cells. Ann. N. Y. Acad. Sci. 1042, 246-254.

(40) Magalhaes, H. I., Wilke, D. V., Bezerra, D. P., Cavalcanti, B. C., Rotta, R., de Lima, D. P., Beatriz, A., Moraes, M. O., Diniz-Filho, J.,

DOI: 10.1021/acs.chemrestox.5b00123
Chem. Res. Toxicol. XXXX, XXX, XXX-XXX

EPA-HQ-2018-000065-0000525

and Pessoa, C. (2013) 4-Methoxyphenyl)(3,4,5-trimethoxyphenyl)-methanone inhibits tubulin polymerization, induces G2/M arrest, and triggers apoptosis in human leukemia HL-60 cells. Toxicol. Appl. Pharmacol. 272, 117 - 126.

(41) Cocca, C., Dorado, J., Calvo, E., Lopez, J. A., Santos, A., and Perez-Castillo, A. (2009) 15-Deoxi-Delta(12,14)-prostaglandin J_2 is a tubulin-binding agent that destabilizes microtubules and induces mitotic arrest. Biochem. Pharmacol. 78, 1330 - 1339.

DOI: 10.1021/acs.chemrestox.5b00123
Chem. Res. Toxicol. XXXX, XXX, XXX - XXX