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Cytotoxicity of Thirdhand Smoke and Identification of Acrolein as a Volatile Thirdhand Smoke Chemical That Inhibits Cell Proliferation

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Running title: Toxic VOCs in Thirdhand Smoke

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Abstract

Thirdhand smoke (THS) is a mixture of chemicals that remain on indoor surfaces after smoking has ceased. These chemicals can be inhaled, ingested, or absorbed dermally, and thus could impact human health. We evaluated the cytotoxicity and mode of action of fresh and aged THS, the toxicity of volatile organic chemicals (VOCs) in THS, and the molecular targets of acrolein, a VOC in THS. Experiments were done using mouse neural stem cells (mNSC), human pulmonary fibroblasts (hPF), and lung A549 epithelial cells. THS-exposed cotton cloth was extracted in Dulbecco's Eagle Medium and caused cytotoxicity in the MTT assay. THS extracts induced blebbing, immotility, vacuolization, cell fragmentation, severing of microfilaments and depolymerization of microtubules in mNSC. Cytotoxicity was inversely related to headspace volume in the extraction container and was lost upon aging, suggesting that VOCs in THS were cytotoxic. Phenol, 2',5'-dimethyl furan and acrolein were identified as the most cytotoxic VOCs in THS, and in combination, their cytotoxicity increased. Acrolein inhibited proliferation of mNSC and hPF and altered expression of cell cycle regulatory genes. 24 hours of treatment with acrolein decreased expression of TFDP1, a factor needed for the G1 to S transition in the cell cycle. At 48 hours, WEE1 expression increased, while ANACP1 expression decreased consistent with blocking entry into and completion of the M phase of the cell cycle. This study identified acrolein as a highly cytotoxic VOC in THS which killed cells at high doses and inhibited cell proliferation at low doses.

Key words: thirdhand smoke, THS, volatile organic chemicals, acrolein, cytotoxicity, stem cells, lung cells, cell proliferation, cell cycle

Introduction

Thirdhand smoke (THS) is a dynamic mixture of volatile, semi-volatile, and non-volatile chemicals, most of which are present in mainstream and secondhand tobacco smoke (Matt *et al.*, 2011). As THS ages, chemicals are continually formed and depleted in THS residue through interactions with chemicals in the ambient environment (Petrick *et al.*, 2011; Sleiman *et al.*, 2010). Aging causes a build-up of nicotine derivatives, such as tobacco specific nitrosamines (TSNAs) (Thomas *et al.*, 2014;), as well as the loss of volatile organic chemicals (VOCs) (Sleiman *et al.*, 2010; Sleiman *et al.*, 2014). Therefore the chemical composition of THS residue changes over time, which could impact its effects on biological systems.

While the adverse effects of mainstream and secondhand cigarette smoke are well documented (USDHHS, 2014) and can involve organs other than the respiratory system (DiCarlantonio and Talbot, 1999; Gieseke and Talbot, 2005), the health effects of THS have only recently been explored. THS can damage DNA (Hang *et al.*, 2013), alter differentiation in prenatal rat lung (Rehan *et al.*, 2011), and adversely affect multiple organ systems in mice (Martins-Green *et al.*, 2014). Given its potential to adversely affect health and its wide-spread distribution in indoor environments, THS has emerged as a public health concern, and further work is required to understand its effects on human health.

Human exposure to THS can occur through dermal contact (Hammer *et al.*, 2011), ingestion (especially in infants and toddlers), and inhalation of the VOCs that come off surfaces containing THS residue (Bahl *et al.*, 2014). Inhalation exposure is the most difficult to avoid and could have significant health consequences across all age groups depending on the level and duration of exposure. VOCs in cigarette smoke, such as acetonitrile, dimethyl furan and 2',5'-dimethylfuran, have been reported in indoor environments where smoking occurred (Sleiman *et al.*, 2014). Some chemicals, such as acrolein and acrylonitrile, were present in THS at concentrations higher than those considered safe by the State of California (OEHHA, 2014).

The concentration of VOCs changed rapidly after smoking occurred, possibly due to sorption onto indoor surfaces (Sleiman *et al.*, 2014). These sorbed VOCs desorb gradually into the indoor air and can be inhaled by occupants. It is important to identify those VOCs in THS that are likely to be detrimental to health (Matt *et al.*, 2011) |

The purpose to this study was to test the hypothesis that THS-exposed fabric contains chemicals that impair cell health, and then to identify cytotoxic chemicals in THS extracts and their mode of action. THS collected on terry cloth was extracted in cell culture medium at various times after deposition. The cytotoxicity of these extracts was determined using the MTT assay and the mode of action was evaluated using a live cell imaging assay. Authentic standards of VOCs identified in the headspace of THS extracts were further evaluated using the MTT assay. The mode of action and molecular targets were identified using a live cell imaging assay and gene expression arrays.

Materials and Methods:

Generation of THS: THS was generated in a controlled experimental chamber at the University of California San Francisco (UCSF) as described previously (Bahl *et al.*, 2014; Schick *et al.*, 2014). Terry cloth was exposed to cigarette smoke from Marlboro Red cigarettes for 114 hours over a span of 11 months, while polyester and paper were exposed for 257 hours each over 6 months. We previously estimated the exposure for terry cloth to be equivalent to approximately 133 cigarettes. Using similar calculations ([Bahl *et al.*, 2014](#)), the polyester and paper were exposed to approximately 185 cigarettes. Exposed fabric was wrapped tightly in a plastic bag and stored with virtually no headspace.

Preparation of THS extracts in culture medium: Upon receipt at UCR, THS exposed terry cloth, polyester fleece and paper were stored at room temperature in amber bottles with

headspace. Aqueous extracts of THS were prepared in Dulbecco's Modified Eagle Medium (DMEM). For all extractions, terry cloth, polyester fleece, and paper were weighed and cut into small pieces. A known weight of the fabric (0.125 g of fabric/ml of medium) was soaked in culture medium in 15 ml conical tubes. The tubes were subjected to constant agitation using a rotating shaker for 1 or 2 hours at either room temperature or 4°C. For Figure 1, the tubes were directly centrifuged at 4,000g for 5 minutes; however, for all other data, the contents were transferred to a 3 ml plastic syringe (Sigma-Aldrich, St. Louis, MO) inside a fresh 15 ml conical tube, which was centrifuged at 4,000g for 5 minutes to recover the culture medium absorbed in the fabric. The recovered THS extract was filtered through 0.22µm sterile filters (Pall Corporation, Port Washington, NY), aliquoted into 1.5 ml vials, and stored at -80°C. Some THS extracts of terry cloth were made immediately after receipt at UCR, and these are referred to as "fresh" in this article. Although the terry cloth had been stored at UCSF for 11 months before extraction, it was wrapped tightly in 0.06 mm thick polypropylene film and minimal loss of VOCs was expected during this time. Extracts of terry cloth were also made after 2 and 5 month of aging in amber bottles with head space at UCR. During this aging period, there was an opportunity for VOCs to escape into the headspace. THS extracts from polyester fleece and paper were made after 1 month of storage in amber bottles at UCR.

Identification of VOCs in THS extract headspace: The volatile chemicals in THS headspace were identified at the Lawrence Berkeley National Laboratory using proton transfer reaction mass spectrometry (PTR-MS, IONICON). 1 mL of the THS extract was placed in a 20 mL glass impinger, with the inlet connected to a clean air flow of 100 mL/min and the outlet connected online to the PTR-MS and to a Tenax sorbent tube. PTR-MS mass spectrometry scan was performed in the range of m/z 18 - 150. The sampling flow for the Tenax sorbent tube was 20 mL/min, for duration of 1 hour. After sampling, the sorbent tube was analyzed using a

Gerstel thermal desorption system coupled with a GC-MS (Agilent 6890, MSD5973) under operational parameters reported previously (Destailats *et al.*, 2006).

Preparation of solutions of authentic standards of VOC: Twenty-five authentic standards of VOC (Table 1) were purchased (Fisher Scientific, Tustin, CA) then prepared in phosphate buffered saline (PBS) at stock concentrations of 0.01M. These chemicals were chosen for testing for three reasons: (1) some were identified in THS (Ueta *et al.*, 2010), (2) some were identified in the air of American homes with THS, and (3) some were identified in the headspace of our THS extracts made after 2 months of aging in amber bottles. Working concentrations of each standard were prepared in the appropriate culture medium through serial dilutions and tested in the MTT or a live cell imaging assay.

In-vitro models for evaluating toxicity: In vitro models, which are rapid, predictive, and cost effective, are valuable for toxicity testing (Talbot, 2008). Whole THS and individual chemicals were screened using mNSC, a model for neonatal brain, which is adversely affected in humans by cigarette smoke (Bruin *et al.*, 2010; Dwyer *et al.*, 2009). Follow-up assays were performed using human pulmonary fibroblasts (hPF) and lung epithelial cells (A549), which model the adult lung, a major target of inhaled VOC from THS. This choice of cells allowed comparison of the sensitivity of neonatal and adult cells to THS.

Culturing mouse neural stem cells (mNSC): mNSC were cultured in Dulbecco's Modified Eagle's Medium (DMEM) (Lonza, Walkersville, MD) containing 10% fetal bovine serum (Sigma-Aldrich, St. Louis, MO), 5% horse serum (Invitrogen, Grand Island, NY), 1% sodium pyruvate (Lonza, Walkersville, MD) and 1% penicillin–streptomycin (GIBCO, Invitrogen, Carlsbad, CA) (Behar *et al.*, 2012). The cells were cultured in Nunc T-25 tissue culture flasks (Fisher Scientific, Tustin, CA) at 37°C in 5% CO₂ and 95% relative humidity with medium changes on alternate days. When cells reached 80% confluency, they were

used in an experiment. For detachment, cells were washed with Dulbecco's phosphate buffered saline (DPBS) then treated with 0.05% trypsin EDTA/DPBS (GIBCO, Invitrogen, Carlsbad, CA) for 1 min at 37°C. For experiments, cells were plated using the protocol described previously (Bahl *et al.*, 2012) at 2500 cell/ well (7,812 cells/cm²) in 96 well plates for the MTT assay and at 5000 cell/ well (2,631 cell/cm²) in 24 well plates for live imaging.

Culturing hPFs. hPF (ScienCell, Carlsbad, CA) were cultured using the suppliers' protocol (Bahl *et al.*, 2012) in complete fibroblast medium containing 2% fetal bovine serum, 1% fibroblast growth serum, and 1% penicillin-streptomycin. hPFs were grown on poly-L-lysine (ScienceCell, Carlsbad, CA) (15 µl/10 ml) coated T-25 flasks, which were prepared and incubated overnight prior to use. hPF were examined microscopically daily, and medium was changed every other day. hPF were cultured in 5% CO₂ at 37°C and 95% relative humidity until 85% confluent, at which time they were used for MTT testing. For sub-culturing and experimental set up, cells were washed with DPBS and detached with 0.01% trypsin diluted in DPBS for 1 min at 37°C after which FBS (Sigma-Aldrich, St. Louis, MO) was added to neutralize trypsin. Cells were plated at 5,000 cells/well (15,625 cell/cm²), in 96 well plates, for the MTT assay and at 10,000 cells per well (5,263 cells/cm²), in 24 well plates, for live cell imaging.

Culturing A549 lung epithelial cells: A549 cells (ATCC, Manassas, VA), which are widely used for toxicity testing (Bakand *et al.*, 2009), were grown on non-coated T-25 flasks and cultured in F-12K medium (with L-Glutamine) with 10% A549 Specific FBS in 5% CO₂ at 37°C and 95% relative humidity. A549 cells were examined microscopically daily until 85% confluent, at which time they were removed from the flask using 0.025% trypsin diluted in DPBS for 2 minutes at 37°C. After 2 minutes of incubation with trypsin, cells were checked using a microscope, and the flask was tapped to loosen cells if necessary. When cells were detached, 3 ml of FBS (Sigma-Aldrich, St. Louis, MO) were added to neutralize trypsin. For the MTT assay, cells were plated at 15,000 cells/well (46,875 cells/ cm²) in 96 well plates.

Testing THS extracts and VOCs for cytotoxicity using mNSC, hPF, and A549 cells

in the MTT assay: The MTT assay relies on conversion of a tetrazolium salt called 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) into an insoluble purple colored formazan by the action of mitochondrial reductases (Mossman, 1983). The purple formazan can be dissolved in DMSO, and its absorbance is recorded at 570 nm, which is a measure of the metabolic activity of cells. A higher absorbance reading indicates a higher metabolic activity. The metabolic activity and therefore the absorbance decreases with reduction in cell survival. Cells were plated in 96-well plates for 24 hours, then various concentrations of THS extract or authentic VOCs were added to wells, and plates were incubated for another 48 hours at 37°C and 95% relative humidity. Untreated wells adjacent to the cells with the lowest concentration of treatment served as the negative control. A vapor control column of untreated cells was also plated adjacent to the wells with the highest concentration treatments (Bahl et al., 2012). At the end of incubation, MTT (Sigma–Aldrich, St. Louis, MO) prepared at 5 mg/ml in DPBS with calcium and magnesium (Fisher Scientific, Tustin, CA) was added to each well, and plates were incubated for 2 hours at 37°C. Plates were drained of solution, and 100 µL of dimethyl sulfoxide (DMSO) (Fisher, Tustin, CA) were added and mixed by pipetting to form a uniformly colored solution. Absorbance was read at 570 nm using the Epoch Microplate Spectrophotometer (Biotek, Winooski, VT). Each experiment was performed three times and duplicate wells were run for each group in each experiment. Similar experiments were done using hPF and A549 cells in the MTT assay with chemicals that were cytotoxic to mNSC and chemicals in the DALYs list (Sleimen *et al.*, 2014). VOCs identified in our THS headspace samples and those reported by Ueta *et al.* (2010) were also tested on mNSC by replacing the dose every 4 hours for up to 48 hours to determine if replenishing VOCs frequently would shift the dose response curves. Combinations of VOCs were also tested on mNSC to determine if their toxicity is additive or synergistic.

Live cell imaging: mNSC or hPF were plated in 24-well plates and incubated at 37°C for 24 hours. Varying concentrations of THS or acrolein were prepared in culture medium and added to plated cells, which were transferred to a Nikon BioStation CT incubator (Nikon, Melville, NY) and imaged every 1 hour (THS) or 2 hours (acrolein) for 48 hours. For each well, data were collected from five different fields. Cell proliferation was analyzed in time-lapse images using video bioinformatics protocols created in CL-Quant software (DRVision, Seattle, WA). Each protocol was verified by comparing ground truth obtained using ImageJ to data analyzed with CL-Quant. For proliferation, the results of ground truth and CL-Quant analyses were 95-98% similar.

Fluorescent staining for actin and tubulin: mNSC were plated on chamber slides (Ibidi, Planegg, Germany) at 2500 cells/well and incubated for 24 hours at 37°C. Cells were treated with THS extracts for 2 hours, and then fixed using freshly prepared 4% paraformaldehyde. Cells were stained for tubulin using an anti-tubulin antibody (Cell Signaling Technologies, Danvers, MA) and for actin using phalloidin conjugated to Alexa fluor 488, (VWR, Radnor, PA) for 1 hour at room temperature. After washing with PBS to remove unbound phalloidin-Alexa fluor 488 and anti-tubulin antibody, mounting medium containing DAPI (Vector Laboratories Burlingame, CA) was added, and cells were imaged using a Nikon Ti Eclipse fluorescent microscope (Nikon, Tokyo, Japan).

RT² PCR Profiler Array: hPF were plated in 6 well plates at 50,000 cells/well (5,263 cells/cm²) and allowed to attach overnight at 37°C for 24 hours. After 24 hours, cells were treated with 1μM acrolein for 4, 24, 48 and 72 hours after which RNA was isolated using the Qiagen RNAeasy plus mini kit (Qiagen, Valencia, CA). RNA was checked for degradation using the Agilent 2100 Bioanalyzer, and only those samples having an RIN (RNA integrity number) of 7 or above were used for further processing. 400ng of RNA from each sample was used to prepare cDNA using the Qiagen RT² First Strand Kit (Qiagen, Valencia, CA). cDNA was

amplified through a PCR reaction using primers for GAPDH to make sure that cDNA synthesis reaction has worked. Primer sequences used were as follows: 5'-GGAGccAAAAGGGTCATCATC-3' (forward) and 5'-AGTGATGGCATGGACTGTGGT-3' (reverse).

The effect of THS on the expression of 84 genes associated with the cell cycle was evaluated using the Qiagen Human Cell Cycle RT² Profiler Array. The reaction mixture was prepared by mixing cDNA with Qiagen RT² SYBR Green FAST Mastermix according to the manufacturer's protocol and then loaded on to the array plate. qPCR was performed using the BIO-RAD CFX384 Real Time PCR detection system (Bio-Rad, Hercules, CA). Ct values were entered into the online Qiagen data analysis center to obtain fold change values for gene expression.

Follow up PCR: Primers were designed for those genes whose levels were significantly altered across the three RT² PCR profiler array plates to confirm their changes in expression levels. Qiagen HotStarTaq Master Mix (Qiagen, Velencia, CA) was used to run PCR reactions using the BioRad Thermal Cycler (BioRad, Hercules, CA). Primers used were as follows: CASP3: 5'-TGAAACAGTATGCCGACAAG-3' (forward) and 5'-TAGAGTTCTTTTGTGAGCATG-3' (reverse); TFDP1: 5'-TTACCAAACGAGTCAGCTTAT-3' (forward) and 5'-CCTCTGTCTTTCCACCTCTAAG-3' (reverse); ANAPC2: 5'-AAAGGTTCTTCTACCGCATCTA-3' (forward) and 5'-TGGCAGAGATATAGAGGGTGAT-3' (reverse); WEE1: 5'-TATGAAAGTCCCGGTATACAACA-3' (forward) and 5'-ATCGAACTACATGAGAATGCTG-3' (reverse). Lonza DNA FlashGels were run to separate PCR products which were imaged using a Lonza FlashGel imaging system (Lonza, Walkersville, MD).

Statistical Analysis: For the MTT assay, statistical analyses were done on the raw data using GraphPad Prism software. Group means were compared using a one-way ANOVA. When $p < 0.05$ in the ANOVA, Dunnett's post hoc test was used to compare each dose to the control. For graphing, MTT absorbance data were normalized to 100% and treatment groups were expressed as a percentage of the negative control. IC_{50} s were determined from dose response curves using non-linear regression analysis in GraphPad Prism (Bahl *et al.*, 2012). In cases where GraphPad could not derive an IC_{50} , the value was read off the dose response curve. The lowest observed adverse effect levels (LOAEL) (lowest dose that was significantly different than the control) and the no observed adverse effect levels (NOAEL) (the highest concentration that was not significantly different than the control) were determined.

For live cell imaging analysis, the percent area (confluency) was normalized to the area of the first frame (2 hours). 4 or 5 videos were analyzed for each treatment within every experiment, and three experiments were used for statistical analysis. Two-way ANOVA was performed by comparing area (confluency) at each time to the 4 hour data to determine if there was a significant effect of concentration and time of treatment on area (confluency). The percentage of dead cells were analyzed using a one way ANOVA as described above with each group being compared to the untreated control using Dunnett's posthoc test.

For RT-PCR data, raw Ct values were put in the Qiagen data analysis center to identify those genes with expression levels that were significantly different than the control.

Results:

Extraction conditions affect the cytotoxicity of THS in the MTT assay: The cytotoxicity of THS extracted from terry cloth soon after receipt from USCF depended on the time and temperature of extraction (Fig. 1A). At an extraction ratio of 0.125g of fabric/ml of medium, maximum cytotoxicity was observed at 30% and 100% concentrations after 1 hour of

extraction at room temperature. Cytotoxicity was lost at the 30% concentration in samples extracted for 2 hours at room temperature. The extracts prepared at 4°C for 1 hour were not cytotoxic at any concentration tested and were weakly cytotoxic at the 100% concentration when extracted for 2 hours at 4°C (Fig. 1A).

Headspace volume of the extraction vessel was inversely correlated with cytotoxicity of the extracts (Fig. 1B). Extracts made for 1 hour at room temperature in tubes with 15 ml of headspace were cytotoxic at both the 30% and 100% concentrations. In contrast, extracts made for 1 hour at room temperature in T-25 flasks with 70 ml of headspace were cytotoxic only at the 100% concentration. The 2 hour extract was less cytotoxic than the 1 hour extract, and the smaller the headspace, the more potent the 2 hour extract (Fig. 1B).

Aging of THS decreased the cytotoxicity of the extracts: THS-exposed terry cloth was aged in amber bottle with 250 ml of headspace for 5 months after receipt from UCSF, and then THS was extracted at room temperature for 1 and 2 hours using an extraction ratio 0.125g of fabric/ml of medium. Neither of the extracts from terry cloth aged in amber bottles for 5 months was cytotoxic to mNSC in the MTT assay, in contrast to the extracts made soon after receipt from USCF, which were both cytotoxic (Fig. 1C).

Passage number does not affect sensitivity to THS: To determine if cell passage number affects response to THS, extracts made from polyester fleece and paper, 2 months after receipt from UCSF were tested for cytotoxicity using early and late passages of mNSC. Extracts were made at room temperature for 2 hours using an extraction ratio of 0.25g of sample/ml of medium. For extracts from both polyester and paper, the cells passaged 70-78 times were slightly more sensitive than those passaged 20-25 times (Fig. 1D). However, the differences between the young and old passages were not significantly different at the 100% concentration

for either material when compared using an unpaired t-test ($p = 0.9632$ for polyester and $p = 0.9591$ for paper).

THS affected multiple cell processes: The effects of THS extract on dynamic cell processes were evaluated using live cell imaging coupled with video bioinformatics analysis (Fig. 2). mNSC were treated with 10% and 30% concentrations of THS extracted from terry cloth that was cytotoxic in the MTT assay. Cells treated with 10% THS extract grew at the same rate as control cells, reaching about 90% confluency by 45 hours, whereas cells treated with 30% THS did not grow (Fig. 2A). In the 30% THS treatment group, confluency decreased at 3 hours and remained below the starting value throughout incubation (Fig. 2A). Cells in the 30% group rounded up and appeared dead by 30 hours (Fig. 2B). However, video analysis showed that the round cells could go through cycles of attaching, spreading and rounding up and sometimes rounded up and blebbed (Fig. 2B) (Guan et al., 2014). Cells treated with 30% THS often fragmented and lost a portion of their cytoplasm. This occurred when the cell adhered tightly to the culture dish, and as the main body of the cell moved, the cell was torn into two fragments with the larger fragment usually containing the nucleus (Fig. 2C). Some cells treated with 30% THS had vacuoles in their cytoplasm, consistent with stress (Fig. 2D). The motility of mNSC treated with 30% THS extract was significantly decreased when compared to untreated controls (Fig. 2E). Impaired motility was caused by disruption of the actin microfilaments and depolymerization of the microtubules and was sometimes accompanied by a change in morphology or rounding up of cells (Fig. 2F-G). None of the above effects occurred when mNSC were treated with THS extracts that had aged in amber bottles and lost their cytotoxicity in the MTT assay (shown for cytoskeletal effects in Figs 2H-I).

VOCs identified in THS headspace in this and in other studies: The above data are consistent with the idea that cytotoxicity was caused by VOCs in the THS extracts. Data on VOCs in the headspace of THS extracts and homes were compiled (Table 1). Eleven VOCs

were identified in the headspace of vials containing THS extracts from terry cloth that had aged 3 months at UCR in amber bottles, by which time extracts may not have been cytotoxic. The concentrations of these VOCs ranged from 1.05 to 14.41 $\mu\text{g}/\text{m}^3$. Some VOCs found in terry cloth extracts overlapped with VOCs found in the THS from the homes of cigarette smokers (Table 1, Sleiman *et al.*, 2014). Table 1 also shows the concentrations of VOCs identified in THS from a Japanese study (Ueta *et al.*, 2010), and the DALYS (disability adjusted life years) for VOCs in THS based on data in Sleiman *et al* (2014).

Identification of cytotoxic VOCs using three cell types: All VOCs in Table 1 were screened in the MTT assay using mNSC (Fig. 3 and Supplementary Fig. S2). Only phenol, 2',5'-DMF, and acrolein were cytotoxic to mNSC (Fig. 3).

As a follow-up, phenol, 2',5'-DMF and the VOCs with highest DALYS were also tested using hPF and A549 cells (Fig. 3 and Supplementary Fig. S3). Only phenol, 2',5'-DMF, and acrolein were cytotoxic to all three cell types, at the concentrations tested. Acrolein was the most potent (Fig. 3). In general, the mNSC and hPF cells were more sensitive to the three chemicals than the A549 cells. The IC_{50} s for each chemical and each cell type are given in Table 2.

To determine if the evaporation of the volatile chemicals from the culture media caused underestimation of cytotoxicity, chemicals were replaced every 4 hours instead of at 24 hours (Fig. 4 and Supplement Fig. 2). None of the chemicals tested were significantly more cytotoxic when replaced every 4 hours. The IC_{50} for 2',5'-DMF replaced every 4 hours = 2×10^{-4} M and replaced at 24 hours = 4×10^{-4} M. The IC_{50} for acrolein replaced every 4 hours = 3×10^{-6} M and replaced at 24 hours = 5×10^{-6} M (Fig. 4).

Effect of mixtures of cytotoxic chemicals on mNSC: mNSC were treated with combinations of chemicals identified by Ueta *et al.* (2010) and identified in the headspace of our

THS extracts. These combinations were not cytotoxic at the doses tested (Supplementary Fig. S4). As a follow-up, acrolein, 2',5'-DMF and phenol, which were cytotoxic when tested individually, were tested in combination with mNSC and hPF (Fig. 5), and a significant increase in potency was observed. For mNSC, the IC_{50} of the combination (4.95×10^{-6} M) was very close to that of acrolein alone (3.416×10^{-6} M); however, there was a significant effect at the 3×10^{-7} and 10^{-6} M concentrations, which was not seen with the individual chemicals. For hPF, the IC_{50} for the combination of chemicals was 1×10^{-6} M, which was lower than the IC_{50} for any of the chemicals alone (Fig. 5B).

Acrolein affected cell proliferation but not blebbing, fragmentation, vacuolization or motility of mNSC and hPF: Because acrolein was the most potent of the VOCs studied, follow-up experiments were done to determine its mode of action. mNSC were incubated in a BioStation CT and time-lapse images were recorded every 2 hours for 46 hours (Fig. 6A). At the 10^{-5} M acrolein concentration, about 80% of the cells were dead and the growth curve was flat (Figs. 6B). Analysis of video data showed that death at 10^{-5} M acrolein was caused by apoptosis. Cells that died detached from the dish, rounded up, blebbed, and formed apoptotic bodies (Supplementary Fig. 5). At the 3×10^{-6} M and 10^{-6} M concentrations, cell death was not significantly different from the untreated control but % area was significantly lower (Figs. 6B and C). These observations are consistent with the idea that the lower confluency in these groups was due mainly to a decrease in cell proliferation.

Acrolein produced similar effects on hPF in the live cell imaging assay (Fig. 6D). There were fewer cells in all concentrations of acrolein at 48 hours of treatment (Fig. 6D). Acrolein at 6×10^{-6} M killed 100% of the cells and the growth curve was flat (Fig. 6E). At the 10^{-6} M concentration, there was no cell death (Fig. 6F) but growth (% area Fig. 6E) was significantly lower than the untreated control, indicating acrolein inhibited cell proliferation at this concentration.

Acrolein altered expression of genes involved in cell cycle regulation: No changes in gene expression over 1.5 fold were observed in two pathway arrays run with samples treated 4 hour with 10^{-6} M acrolein, consistent with the effect on growth not occurring at this time (Fig. 6E). However, after 24 hours of exposure to acrolein, expression levels of caspase 3 (CASP3) and transcription factor Dp-1 (TFDP1) were significantly decreased by 2.42 and 7.2-fold, respectively. At 48 hours, the WEE1 G2 checkpoint kinase was significantly increased by 8.6-fold, while expression of the gene for anaphase promoting complex subunit 2 (ANAPC2) was significantly decreased by 2-fold (Fig. 7A). Relative changes in the expression levels of these genes were confirmed by PCR (Fig. 7B).

Discussion:

This study is one of the first to evaluate the cytotoxicity of THS, determine the effect of aging on THS's cytotoxicity, and identify cytotoxic VOCs in THS. Our main findings are summarized in Figure 8. THS extracted from unaged terry cloth was toxic to mNSC causing cell death, blebbing, fragmentation, cytoskeletal disruption, and vacuolization. Loss of cytotoxic activity in fabric samples stored in amber jars with headspace indicated that VOCs are responsible for these effects. Of the VOCs tested, acrolein was the most cytotoxic, causing cell death at high concentrations and inhibiting cell proliferation at low concentrations. Acrolein altered expression of genes that regulate the cell cycle in a manner consistent with reduced proliferation and specifically blocked transition from G1 to S and from G2 to M.

The experiments with different methods of extraction and aging in amber bottles indicated that VOCs in THS were likely responsible for the observed toxicity. This is a reason for concern since inhalation of VOCs in a THS environment is difficult to avoid. The degree of cytotoxicity of THS extracts depended on the temperature and time of extraction. The decreased cytotoxicity of extracts made at 4°C is likely due to slower release of THS chemicals at the lower

temperature. The reduced cytotoxicity of extracts prepared over 2 hours and those prepared in flasks with larger headspace are consistent with the conclusion that cytotoxicity was due to VOCs that are lost during prolonged extraction or when headspace volume is increased.

The idea that VOCs contribute to cytotoxicity was also supported by the observation that THS extracts lost their cytotoxicity upon storage for 5 months in amber bottles with head space, while the concentrations of nicotine, most tobacco alkaloids, and TSNAs did not decrease significantly during storage (Bahl *et al.*, 2014). While some VOCs may have been lost from terry cloth during the first 11 months of storage when they were tightly wrapped in plastic, the rate of loss was likely low as there was little headspace for desorption. From 11 months onwards, terry cloth was stored unwrapped in glass amber bottles with increased headspace which would have facilitated the loss of VOCs. The THS extracts used in this study were from fabric that had aged without new cigarette smoke being deposited on the fabric. In a real indoor environment with smoking taking place, THS would not necessarily become less cytotoxic with time because additional cigarette smoke would be deposited periodically. This could lead to a build-up of higher levels of VOCs and novel compounds such as nitrosamines (Sleiman *et al.*, 2010), which may in turn lead to a greater effect on cellular targets.

Three THS VOCs, phenol, 2',5'-DMF, and acrolein, were cytotoxic to each cell type in the MTT assay. Since a person in a THS environment would be exposed to these chemicals simultaneously, they were tested in combination and were toxic at lower concentrations. Therefore the dose response data for individual VOCs underestimates the actual cytotoxicity in THS. VOCs were screened only with the MTT assay, which evaluates the metabolism of a tetrazolium salt by mitochondrial reductases (Mosmann, 1983), and THS VOCs may have additional effects on cell health, such as damage to DNA, which would not be detected by this assay. While our study focused on 25 VOCs, there are other VOCs in THS (Sleiman *et al.*, 2014), which may also be cytotoxic.

mNSC and hPF were more sensitive than A549 cells to phenol and acrolein, while hPF were more sensitive to 2',5'-DMF than mNSC and A549. This demonstrates the importance of screening multiple cell types when evaluating cytotoxicity. Because hPF were more sensitive to cytotoxic THS chemicals than A549 cells, they are an important *in vitro* model for toxicological studies dealing with adult lung.

Acrolein, a strong electrophile that reacts with proteins and DNA (Moghe *et al.*, 2015), was the most cytotoxic of the VOCs tested. Acrolein adversely affects mitochondrial function, inflammation, and ciliary beating in multiple cell types (Moghe *et al.*, 2015; Talbot *et al.*, 1998). In our study, live cell imaging showed that cell death alone could not account for decreased growth (% area) at low acrolein concentrations and that inhibition of cell proliferation was the mode-of-action of acrolein at non-lethal doses.

The cell cycle molecular targets of acrolein were identified using gene expression arrays. Acrolein is highly reactive and probably starts affecting molecular targets soon after exposure (Thompson and Burcham, 2008a), triggering a cascade of events that leads to decreased expression of caspase 3 (a protease) and TFDP1 (a transcription factor that dimerizes with E2F). Inhibition of caspase 3 and down regulation of TFDP1 arrest the cell cycle (Hashimoto *et al.*, 2011; Yasui *et al.*, 2003) and would block progression from G1 to S (TFDP1) and from G2 to M phase (caspase 3). At 48 hours, expression of WEE1, which blocks transition from the G2 to M phase (McGowan and Russel, 1995), was increased, while expression of ANAPC2, which promotes transition from metaphase to anaphase (Reddy *et al.*, 2007), was decreased by acrolein. Thompson and Burchan (2008) reported genome wide transcriptional responses of A549 cells to acrolein with a greater number of genes affected than in our study. We used 1 μ M acrolein with primary pulmonary fibroblasts, while Thompson and Burchan (2008) used a 100 μ M acrolein with A549 cells. These differences likely account for the difference in the number of genes affected in the two studies. Our data clearly show effects on cell cycle regulatory genes

consistent with inhibition of cell proliferation at concentrations 100- fold lower than that used with A549 cells (Thompson and Burcham, 2008a).

It is important to consider how the effective concentration of acrolein *in vitro* compares to the dose a human receives in a THS environment. In our *in vitro* study, hPF proliferation was inhibited by 10^{-6} M acrolein (Fig. 6D, E). Because acrolein reacts rapidly with proteins in the culture medium, only a fraction of it is available to affect cultured cells (Thompson & Burcham, 2008b). Based on values reported by Thompson & Burcham (2008b), we estimate that the concentration of acrolein actually available to cells in our studies was not 10^{-6} M, but between 10^{-9} and 10^{-8} M.

The acrolein dose for nonsmokers exposed to THS will vary with factors such as the frequency of indoor smoking, ventilation, and time spent in a THS environment, and would likely be lower than in active smokers. Acrolein concentration reaching the respiratory system in a person exposed to THS can be estimated using the data of Sleiman *et al.* (2014). Acrolein concentration in a THS environment was $7\mu\text{g m}^{-3}$, 2 hours after smoking six cigarettes (Sleiman *et al.*, 2014). Considering a tidal volume of 500 ml, an adult would inhale roughly $4.2\mu\text{g}$ of acrolein in 1 hour, assuming 20 inhalations/minute. If all the inhaled acrolein dissolves in the extravascular lung water (EVLW), which is 3.8ml/ kg body weight (Wallin *et al.*, 1994), a 50 kg person would have about 39×10^{-9} M of acrolein in the EVLW. While this value is an overestimation (some acrolein would be exhaled and some would turnover during 1 hour), it nevertheless supports the idea that a person in a THS environment could be exposed to enough acrolein (10^{-9} and 10^{-8} M based on our *in vitro* studies) to inhibit cell proliferation if not outright kill cells.

There were important differences in the responses of mNSC to 30% THS extracts and 10^{-6} M acrolein. While non-lethal concentrations of both THS and acrolein inhibited proliferation, only THS also caused blebbing, fragmentation, vacuole formation, loss of motility, disruption of

microfilaments, and depolymerization of microtubules. The chemicals in THS responsible for the latter effects have not yet been identified and will be the subject of future studies.

In summary, our data support the idea that inhalation of THS VOCs has adverse effects on human health. Lung fibroblasts, which were killed by high doses of THS and acrolein and inhibited from dividing by low doses, perform important functions, such as secretion of the extracellular matrix (McAnulty, 2007) and alveolar regeneration (Plantier *et al.*, 2007). A loss of lung fibroblasts due to cell death or decreased proliferation could disrupt lung homeostasis and lead to conditions such as pulmonary emphysema (Togo *et al.*, 2008). While the concentrations of THS and acrolein in the brains of smokers and humans exposed to THS are not known and are likely less than that in the lung, neural stem cells were similarly affected by both THS and acrolein. An infant or small child in a THS environment is likely to take up higher levels of acrolein per unit of body mass than an adult, and toxicants can accumulate at a higher concentration in the brain of infants due to less efficient metabolism and clearance from the body (Ginsberg *et al.*, 2004). These factors could have serious implications on the postnatal brain, which continues to develop throughout adolescence (Stiles and Jernigan, 2010).

Supplementary Data description:

Supplementary Fig. 1. A comparison of area (confluency) evaluated using either CL-Quant software or ground truth analysis (obtained by hand-tracing the area of the cells using ImageJ software). (A) Ground truth for control cells. (B) Ground truth for cells treated with 30% THS. Ground truth results validate the CL-Quant protocol. Each point is the mean of two experiments.

Supplementary Fig. 2. MTT assay data for 13 VOCs identified in the headspace of THS extracts tested on mNSC. Each chemical was tested in two separate experiments which were

then averaged. Chemicals were replaced every 24 hours and every 4 hours in two separate sets of experiments. Absorbance data for each dose are plotted as a percent of the untreated control. Each graph represents mean \pm SEM of two experiments.

Supplementary Fig. 3. MTT assay data showing the effects of chemicals with the highest number of DALYs on mNSC, hPF and A549 cells. Each graph represents mean \pm SEM of two experiments.

Supplementary Fig. 4. MTT assay data showing the effect of combinations of VOCs on mNSC. (A) Benzene, toluene, ammonia and pyrrole were tested in combination. (B) Acetonitrile, 3-ethylenepyridine, toluene, phenol, benzaldehyde, acetophenone, quinoline, benzonitrile, acetone, 2-pentanone, 2-butanone and 1H-Pyrrolo[2,3-b]pyridine 2-methyl- were tested in combination. Each graph represents mean \pm SEM of two experiments.

Supplementary Fig. 5. Film strip of cells undergoing apoptosis when treated with 10^{-5} M acrolein. Cells detached from the substrate, rounded-up, blebbed, and shed apoptotic bodies. Arrow indicates an apoptotic body that has detached from the cell.

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Conflict of interest: None

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Figure Legends

Figure 1. **Factors affecting THS cytotoxicity in the MTT assay.** MTT dose-response curves showing absorbance (percent of control) plotted as a function of the THS extract concentration. mNSC were used in all experiments. (A) Effect of temperature and length of extraction on cytotoxicity of THS. (B) Effect of headspace volume on cytotoxicity of THS. (C) Effect of aging 5 months in amber bottles with headspace on cytotoxicity of THS. (D) Effect of passage number on cytotoxicity of THS. Samples designated “fresh” were extracted from terry cloth immediately upon receipt from UCSF without aging in amber bottles. Each point is the mean \pm SEM of three or four experiments. In A, B, and C, each dose was compared by ANOVA to the untreated fabric extract control. In D, the passage 20 and passage 70 data for each fabric were compared using a t-test at the 100% dose. * = $p < 0.05$; ** = $0.001 < p < 0.01$; **** $p < 0.0001$; ns = not significant.

Figure 2. **Effects of THS on confluency, morphology, and motility of mNSC.** mNSC were treated with THS extracted from terry cloth upon receipt of samples from UCSF, and cells were imaged live for 48 hours. (A) Dose-response curves showing area (confluency) vs. time. Data are plotted as means \pm SEM are for three experiments. (B) Micrographs showing rounding and bleb formation characteristic of cells treated with 30 % THS. (C) Sequence of micrographs of a mNSC treated with 30% THS extract showing fragmentation during treatment. (D) Example of a mNSC treated with 30% THS that had vacuoles in its cytoplasm. (E) Sequence of images showing motility of control and treated (30% THS) mNSC; the control cells were motile over the 4 hour interval, while treated cells moved very little. Circles show the same cell at different times. (F) Images showing the effect of fresh THS extracts on actin microfilaments (green) and (G) microtubules (red) in mNSC. (H) Images showing that THS extracts aged in amber bottles with headspace for 5 months lost their ability to affect actin microfilaments and microtubules.

Figure 3. **(A) Phenol, (B) 2',5'-DMF and (C) acrolein were cytotoxic to mNSC, hPF, and A549 cells in the MTT assay.** Absorbance (percent of control) is plotted as a function of chemical concentration. Each curve represents means \pm SEM for three experiments. Each concentration was compared by ANOVA to the untreated control. * = $p < 0.05$; ** = $0.001 < p < 0.01$; *** = $0.0001 < p < 0.001$; **** = $p < 0.0001$.

Figure 4. **Effect of replacement of VOC on cytotoxicity to mNSC.** (A) phenol, (B) 2',5'-DMF and (C) acrolein were replaced every 4 hours and tested on mNSC in the MTT assay. Absorbance (percent of control) is plotted as a function of chemical concentration. Each point represents the mean \pm SEM for four values from two experiments. Each concentration was compared by ANOVA to the untreated control. Absorbance values for the two highest concentrations were compared in the two experiments using a t-test. * = $p < 0.05$; *** = $0.0001 < p < 0.001$; **** = $p < 0.0001$.

Figure 5. **Effect of acrolein, 2',5'-DMF and phenol in combination:** Dose response curves for three VOCs tested in combination on (A) mNSC and (B) hPF. Absorbance (percent of control) is plotted as a function of chemical concentration. Each point represents mean \pm SEM for three experiments. * = $p < 0.05$; ** = $0.001 < p < 0.01$; *** = $0.0001 < p < 0.001$; **** = $p < 0.0001$.

Figure 6. **Effect of acrolein on mNSC and hPF proliferation:** (A) Micrographs showing mNSC confluency after treatment with acrolein for 48 hours. (B) Graph showing confluency of mNSC over 46 hours in control and treatment groups. (C) Graph showing the percentage of dead mNSC at 46 hours in each group. (D) Micrographs showing hPF confluency after treatment with acrolein for 48 hours. (E) Graph showing confluency of hPF over 46 hours. (F) Graph showing

the percentage of dead hPF in each group at 46 hours. For B and E, area (confluency) at each time point is expressed as a percentage of confluency at 2 hours following start of treatment. Each point represents mean \pm SEM for three experiments, each having 4 or 5 videos. For C and F, each bar represents mean \pm SEM for three experiments, with five fields each. * = $p < 0.05$; ** = $0.001 < p < 0.01$; *** = $0.0001 < p < 0.001$; **** = $p < 0.0001$.

Figure 7. 10^{-6} M acrolein decreased expression of TFDP1, CASP3 and ANAPC2 and increased expression of Wee1. (A) Alteration in gene expression observed with RT² PCR profiler array. Each bar is an average of three experiments. (B) PCR products observed with gel electrophoresis. GAPDH was used as the housekeeping (loading) control. (C) Functions of the effected genes.

Figure 8. Diagram summarizing the response of cells to extracts of THS and to individual VOCs in THS: Aqueous extracts of THS caused cell death, blebbing, fragmentation, cytoskeletal disruption and vacuolization in mNSC at the 100% concentration . Phenol, 2',5'-DMF and acrolein caused cell death in mNSC, hPF and A549 cells at 10^{-2} M, 10^{-3} M and 10^{-5} M dose respectively. 10^{-6} M acrolein inhibited cell proliferation in hPF by decreasing expression of TFDP1, caspase 3, ANAPC2 and increasing expression of Wee1.

Table 1. VOCs in THS and their concentrations

Chemical Name	Concentration in headspace sample¹	Concentration in THS aged in an 18 m³ environmental chamber. Sleiman et al²	Concentration reported by Ueta et al³.	DALYs lost per year per 100,000 Sleiman et al
Acrolein		2.4 – 127.9 µg/m ³ 4.2 – 228.4 nM		10-100
Furan		5.6 – 261.3 µg/m ³ 8.23 – 384.3 nM		1-10
Acrylonitrile				0.1 - 1
1',3'-butadiene				0.1
Acetaldehyde				0.1
Isoprene		32.2 – 272.3 µg/m ³ 47.3 – 400 nM		0.01 – 0.1
Benzene		14.5 – 263.5 µg/m ³ 18.5 – 337.8 nM	0 – 4 µg/m ³	0.01 – 0.1
Styrene		0.2 – 129 µg/m ³ 192pM – 124 pM		0.01 – 0.1
Toluene	4.14 µg/m ³ 0.044 nM	15.7 – 226.7 µg/m ³ 17 – 246 nM	0-10 µg/m ³	0.01 – 0.1
Acetonitrile	3.79 µg/m ³ 0.092 nM	19.5 – 73 µg/m ³ 47.5 – 178 nM		0.01 – 0.1
3-ethylenepyridine	14.41 µg/m ³ 0.139 nM			
Phenol	8.83 µg/m ³ 0.093 nM			

1H-Pyrrolo[2,3-b]pyridine, 2-methyl-	7.26 $\mu\text{g}/\text{m}^3$ 0.054 nM	
Benzaldehyde	7.64 $\mu\text{g}/\text{m}^3$ 0.071 nM	1.2 – 26.1 $\mu\text{g}/\text{m}^3$ 1.13 – 24.6 nM
Acetophenone	5.43 $\mu\text{g}/\text{m}^3$ 0.045 nM	
Quinoline	4.04 $\mu\text{g}/\text{m}^3$ 0.03 nM	
Benzonitrile	3.7 $\mu\text{g}/\text{m}^3$ 0.03 nM	
Acetone	2.88 $\mu\text{g}/\text{m}^3$ 0.04 nM	25 – 244.5 $\mu\text{g}/\text{m}^3$ 43 – 420 nM
2-pentanone	2.58 $\mu\text{g}/\text{m}^3$ 0.03 nM	
2-butanone	1.05 $\mu\text{g}/\text{m}^3$ 0.014 nM	
2',5'- Dimethylfuran		3.8 – 95 $\mu\text{g}/\text{m}^3$ 3.9 – 99 nM
Ammonia		8-10 mg/ m^3 0.47 – 0.58 μM
Pyrrole		0.6 – 0.8 mg/ m^3 8.9 – 11.9 nM
2-furaldehyde		
Xylene		

¹Sampled in the current study from terry cloth that had aged 14 months before extraction.

²Data taken from Sleiman et al 2014.

³Data from Ueta et al 2010.

⁴DALYS taken from Sleiman et al 2014.

Table 2: IC₅₀, NOAEL and LOAEL values for acrolein, 2',5'-DMF and phenol

Chemical Name	mNSC	hPF	A549
Acrolein	IC ₅₀ : 6 x10 ⁻⁶ M LOAEL: 3 x 10 ⁻⁶ M NOAEL: 10 ⁻⁶ M	IC ₅₀ : 6 x10 ⁻⁶ M LOAEL: < 6 x10 ⁻⁶ M NOAEL: 3 x 10 ⁻⁶ M	IC ₅₀ : 4 x10 ⁻⁵ M LOAEL: 10 ⁻⁵ M NOAEL: 3 x 10 ⁻⁶ M
2',5'-dMF	IC ₅₀ : 4 x 10 ⁻⁴ M LOAEL: < 4 x 10 ⁻⁴ M NOAEL: 10 ⁻⁴ M	IC ₅₀ : 2 x 10 ⁻⁴ M LOAEL: 10 ⁻⁴ M NOAEL: 10 ⁻⁵ M	IC ₅₀ : 6 x 10 ⁻⁴ M LOAEL: < 6 x 10 ⁻⁴ M NOAEL: 10 ⁻⁴ M
Phenol	IC ₅₀ : 1.1 X 10 ⁻³ M LOAEL: < 1.1 x 10 ⁻³ M NOAEL: 10 ⁻⁴ M	IC ₅₀ : 2 X 10 ⁻³ M LOAEL: 10 ⁻⁴ M NOAEL: 10 ⁻⁵ M	IC ₅₀ : 9 x 10 ⁻³ M LOAEL: 10 ⁻⁴ M NOAEL: 10 ⁻⁵ M