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## Cytotoxicity of eight cigarette smoke condensates in three test systems: Comparisons between assays and condensates

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### ABSTRACT

Cytotoxic properties of tobacco smoke are associated with chronic tobacco-related diseases. The cytotoxicity of tobacco smoke can be tested with short-term predictive assays. In this study, we compare eight mainstream cigarette smoke condensates (CSCs) from commercial and experimental cigarettes in three different cytotoxicity assays with unique and overlapping endpoints. The CSCs demonstrated cytotoxicity in all assays. In the multiple cytotoxicity endpoint (MCE) assay with TK-6 cells, the cigarette varieties that had the highest EC50s for reduced cell growth also showed a positive dose–response relationship for necrotic cells. In the IdMOC multiple cell-type co-culture (MCTCC) system, all CSCs reduced the viability of the cells. Low concentrations of some CSCs had a stimulatory effect in lung microvascular endothelial cells and small airway epithelial cells. In the neutral dye assay (NDA), except for a 100% flue-cured tobacco CSC, there was little consistency between CSCs producing morphological evidence of moderate or greater toxicity and the CSCs with the lowest EC50s in the MCE or MCTCC assays. Overall, cigarettes made with flue-cured tobacco were the most cytotoxic across the assays. When results were expressed on a per-mg of nicotine basis, lower tar cigarettes were the most cytotoxic in primary human respiratory cells.

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### 1. Introduction

The cytotoxicity of tobacco smoke manifests as several pathologic conditions including irritation and inflammation, cell proliferation and hyperplasia, oxidative stress and damage, and decreased organ function, and these conditions may play a role in tobacco-related chronic diseases including emphysema, carcinogenesis, and atherosclerosis (Andreoli et al., 2003; Bombick et al., 1998). The acute effects of cigarette smoking on inflammation and oxidative stress in human, animal, and in vitro models have been reviewed (van der Vaart et al., 2004).

Cytotoxic agents are present in both the gas and particulate phases of cigarette smoke. Hydrogen cyanide, acrolein, and volatile organic compounds are identified as gas phase cytotoxic agents (Thayer and Kensler, 1964; Battista, 1976; Pouli et al., 2003). In the particulate phase, nonvolatile and semi-volatile fractions (especially semi-volatile acidic and neutral fractions) demonstrate cytotoxic activity (Curvall et al., 1984, 1985; Matsukura et al.,

1991). It has been suggested that particulate phase cytotoxicity is due to formaldehyde and residual hydrogen cyanide and acrolein and possibly cyanohydrins formed from the reaction of hydrogen cyanide and aldehydes such as acetaldehyde (Rickert et al., 2007a). However, results as to the relative cytotoxicity of each phase remain contradictory with some reporting that the particulate phase is more cytotoxic in the neutral red assay in CHO cells (Maertens et al., 2009) while others report that the gas phase is more cytotoxic in the neutral red assay with BALB/c 3T3 cells (Tewes et al., 2003).

In vitro cytotoxicity assays are useful in determining the contribution of different tobacco blends or cigarette design characteristics (e.g., filter) to the toxicity of the smoke and also in identifying causative smoke chemicals and mechanistic pathways. Cigarette smoke in the form of particulate phase smoke condensate (Bombick et al., 1997a), gas phase smoke condensate (Bombick et al., 1997b; Pouli et al., 2003), and whole mainstream smoke (Bombick et al., 1997b) are all cytotoxic in a number of in vitro assays. While whole smoke is the closest approximation of the chemical mixture received by a smoker, particulate phase condensate and gas phase condensate allow an investigation of chemicals that

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reside primarily in these fractions. Due to the ease of collection, storage, stability and use with standard assays, mainstream cigarette smoke condensate is perhaps the most routinely used test material for *in vitro* testing; however, it does not provide results equivalent to whole smoke. In one study, the cytotoxicity EC50 for commercial US cigarettes was 8–12 times higher for mainstream smoke condensate than for whole smoke (Bombick et al., 1997a).

Although the cytotoxicity of cigarette smoke has been the subject of published research since at least as early as the 1960s (Thayer and Kensler, 1964; Weiss and Weiss, 1964), and although some have compared a non-commercial reference cigarette in one cell type in several assays (Putnam et al., 2002), none of the previous research has compared the results of a set of diverse cigarette smoke condensates (CSCs) that vary in tobacco blend, novel features associated with claims of reduced smoke emissions, and smoking machine conditions in several *in vitro* cytotoxicity assays with unique and overlapping endpoints. Here we report on the qualitative and quantitative cytotoxicity of eight CSCs prepared from cigarettes that ranged from “low tar” (>6.5–14.5 mg) to “high tar” (>14.5 mg) as determined using the standard International Organization for Standardization (ISO) smoking regimen, commercial cigarette brands described as “light” or “full flavor” by the manufacturer and a commercial 12 mg tar cigarette with a charcoal-containing filter, a reference cigarette (2R4F) blended to be representative of a US standard ISO-regimen “low tar” cigarette, and experimental cigarettes constructed of single-tobacco types. Depending upon the amount of charcoal and the smoking conditions, charcoal filters can reduce levels of gas phase chemicals in cigarette smoke (Polzin, 2008).

The CSCs were tested in the multiple cytotoxicity endpoint (MCE) assay with cultured human lymphoblastoid (TK-6) cells (endpoints: cell growth, apoptosis and necrosis), in primary human microvascular endothelial cells from the lungs, normal human bronchial epithelial cells, and human small airway epithelial cells co-cultured as physically separate cultures interconnected by an overlying liquid medium (the integrated discrete multiple organ co-culture system (IdMOC™; Li et al., 2004; Li, 2008; endpoint: cell viability), and a comparison of visible morphological evidence of cytotoxicity with two strains of cultured mammalian cells (Chinese hamster ovary (CHO) cells and Balb/c-3T3 cells) with endpoints of cell lysis, intracytoplasmic granulation, and cell rounding. CHO and Balb3/t3 cells were compared in this assay owing to their use in previous neutral dye exclusion studies (Bombick et al., 1997b, 1998; NTP, 2003; Putnam et al., 2002).

## 2. Materials and methods

### 2.1. Materials and their sources

Dimethyl sulfoxide (DMSO), >99.9% for molecular biology, was purchased from Sigma–Aldrich (Milwaukee, WI). Cambridge filter pads (CFP) were obtained from Whatman (Maidstone, UK) and used without further purification. Cigarettes for testing were purchased at commercial retail outlets in the Atlanta metropolitan area or were provided by the Massachusetts Department of Public Health. The 2R4F reference cigarettes were purchased from the University of Kentucky Tobacco and Health Research Institute (Lexington, KY). Custom unfiltered, 85 mm research cigarettes containing 100% reconstituted tobacco (“paper” variety reconstituted tobacco), burley, or flue-cured tobaccos were obtained from Murty Pharmaceuticals (Lexington, KY). The experimental cigarettes were constructed with the same cigarette paper used for University of Kentucky reference cigarettes. Table 1 shows the levels of tar, nicotine, and carbon monoxide in the mainstream smoke of these cigarettes and the amount of nicotine in mainstream CSC. All

cigarettes were stored in their original packaging at  $-70\text{ }^{\circ}\text{C}$  until analysis. All bioassays were performed at SITEK Research Laboratories (Rockville, MD).

#### 2.1.1. Smoking conditions

Cigarettes were conditioned, prior to smoking, in an environmental chamber (Parameter Generation Control, Inc., Black Mountain, NC) that was maintained, as specified in ISO 3402:1991, at a temperature of  $22\text{ }^{\circ}\text{C}$  and 60% relative humidity. Cigarette smoke was generated by automated smoking on a linear 16-port ASM 500 smoking machine (Cerulean, Milton Keynes, UK). All cigarettes were smoked to a butt length of either 23 mm for unfiltered cigarettes or the length of the filter tipping paper plus 3 mm for filtered cigarettes.

#### 2.1.2. Preparation of condensate

CSCs were prepared by the CDC as previously reported (Demarini et al., 2008). Briefly, 2–10 cigarettes were machine smoked onto each of four separate CFPs to achieve a combined  $200 \pm 5$  mg of tar as determined from previously reported or measured tar values. Cigarettes were smoked according to either ISO 3308:2000 (35-mL puff volume, 2-s puff duration, 60 s between puffs, and no vent blocking) or according to Massachusetts “intense” parameters (45 mL puff volume, 2 s puff duration, 30 s between puffs, vent holes 50% blocked). Four CFPs were used for all cigarette types to ensure a consistency during extraction and to ensure that no single CFP exceeded its tar capacity of 60 mg.

#### 2.1.3. Extraction

After smoking, the CFPs were collectively inserted into a 14-mL amber vial and extracted with 10 mL of DMSO for 30 min. The extract was then decanted from the vial with aid of a sterile spatula and was saved. Total volume recovered was generally 6 mL. Samples were placed in 1.5-mL amber vials and frozen at  $-20\text{ }^{\circ}\text{C}$  until shipment on dry ice.

### 2.2. Bioassays

#### 2.2.1. Multiple cytotoxicity endpoint assay

The TK-6 cell line was purchased from ATCC (Manassas, VA) and propagated as per their instruction. Hydrogen peroxide and staurosporine were used as positive controls in the absence of metabolic activation. Cyclophosphamide was the positive control in tests with metabolic activation. Metabolic activation consisted of phenobarbital/ $\beta$ -naphthoflavone induced rat liver homogenate (S-9 fraction) mixed with a cofactor pool immediately prior to use (Moltox, Boone, NC). Cells were incubated for 4 h with DMSO (solvent control), positive controls, or CSC with or without metabolic activation. Condensates were tested at 25, 50, 100, 150, or 200  $\mu\text{g}/\text{mL}$ . There were three replicates at each dose level and a successful trial was preceded by one or more preliminary assay(s) to harmonize the assay conditions on two or more independent days. After incubation, cells were washed with phosphate buffered saline (calcium and magnesium free) and allowed to grow for an additional 20 h. Relative cell growth was calculated from the cell count after the final 20-h incubation. Apoptosis and necrosis were determined from Giemsa-stained slides evaluated using a light microscope. A minimum of 500 cells were scored. A normal healthy cell was dark pink in color with a well defined nucleus and cell membrane. Identifying characteristics of apoptotic cells were: cell and/or nuclear shrinkage; nuclear fragmentation; blebbed cell membrane; etc. Identifying characteristics of necrotic cells were: swollen morphology; diffused nucleus; pale color; and, disrupted cell membrane. A CSC was considered to be cytotoxic if it showed a positive dose-response trend and a statistically significant ( $p < 0.05$ ) increase in the percentage of apoptotic or necrotic cells at one or more doses or if

**Table 1**

Yields of tar, nicotine, and carbon monoxide in mainstream smoke and of nicotine in mainstream cigarette smoke condensate (CSC).

Description <sup>a</sup>	Tar (mg/cigarette)	Nicotine (mg/cigarette)	Carbon monoxide (mg/cigarette)	Nicotine (mg/mL condensate)
2R4F reference cigarette (2R4F)	8.9	0.75	12	0.91
Commercial US "light" non-menthol cigarette (LT)	10	0.8	11	1.15
Commercial US "light" non-menthol cigarette (LTMAS)	21.7	1.6	22.8	1.16
Commercial US full-flavor, non-menthol cigarette (FF)	12	0.8	12	0.98
Experimental 100% reconstituted tobacco cigarette (REC)	11.8	0.8	10.8	0.64
Experimental 100% flue-cured tobacco cigarette (BRI)	24.6	5	18.9	2.34
Experimental 100% Burley tobacco cigarette (BUR)	23.3	7.5	11.1	2.33
Commercial US non-menthol cigarette with a charcoal filter (CHAR)	12	1	13	1.09

<sup>a</sup> CSCs 2R4F, LT, FF, REC, BRI, BUR, and CHAR were produced under standard ISO smoking machine conditions; CSC LTMAS was produced under Massachusetts intense smoking machine conditions. CSC LT and LTMAS were generated from the same commercial US "light" non-menthol cigarette.

the relative cellular growth (RCG) was  $\leq 50\%$  for at least two consecutive test concentrations. Treatment-related cytotoxicity was assessed using the Chi-square test. Cochran Armitage Binomial Trend Test was conducted for CSCs that showed a positive response. The study was conducted in compliance with Good Laboratory Practice Standards.

### 2.2.2. IdMOC multiple cell-type co-culture system assay

Human microvascular endothelial cells from the lungs (HMVEC-L), normal human bronchial epithelial cells (NHBE), and human small airway epithelial cells (SAEC) were obtained from Cambrex Bio Science Walkersville, Inc. (Walkersville, MD). Human liver post-mitochondrial supernatant (S<sub>h</sub>-9) was obtained from Molecular Toxicology, Inc. (Boone, NC). Cadmium chloride was used as a positive control. The cells were obtained from the suppliers as monolayer cultures in T-75 tissue culture flasks and were cultured for approximately 3 days before they were used for the study. Twenty-four hours before treatment, the cells were trypsinized from the flasks and plated directly onto IdMOC™ plates (APSciences, Inc., Baltimore, MD) for initial seeding. Cells were incubated on the IDC plates for 24 h in Basal Medium (Basal Epithelial Basal Medium, Small Airway Basal Medium, or Endothelial Basal Medium (Cambrex Bio Science Walkersville, Inc., Walkersville, MD), supplemented with cell-specific growth-factors (Basal Epithelial Growth Medium Single Quot Kit for NHBE, Small Airway Epithelial Cells Single Quot Kit for SAEC, or Essential Growth Medium-Microvascular Single Quot Kit for HMVEC-L, respectively (Cambrex Bio Science Walkersville, Inc., Walkersville, MD), Serum-free DMEM-F12 medium was added to the plates and cells were incubated for a final 24 h in the presence of either CSC or DMSO as solvent control. CSCs were tested at concentrations of 25, 50, 100, or 200  $\mu\text{g}/\text{mL}$  in the presence or absence of S<sub>h</sub>-9. At the end of the final 24 h incubation, cytotoxicity was determined by colorimetric quantitation of blue formazan formed by living cells from enzymatic cleavage of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) dye. On two or more independent days one or more preliminary assay(s) were conducted to harmonize the assay conditions followed by a successful trial. All treatments were performed in triplicate. The concentration required for 50% cytotoxicity (EC<sub>50</sub>) based on MTT cleavage was calculated from the dose–response equation derived by linear regression analysis.

### 2.2.3. Neutral dye assay

A subpassage of the BALB/c-3T3 cell line (clone A31) originally established by Dr. Kakunaga, National Institutes of Health, Bethesda, Maryland, was obtained from the Mobil Oil Corporation (Princeton, New Jersey). The CHO cell line (clone W-B1), originated at Litton Bionetics, was obtained from the Environmental Health Research and Testing Laboratories (Lexington, Kentucky). Standard

positive reference material (SPRM) extracts were segmented polyetherurethane film containing 0.1% since diethyldithiocarbamate (SPRM-A) and segmented polyetherurethane film containing 0.25% zinc dibutyldithiocarbamate (SPRM-B) (Hatano Research Institute, Hadano, Japan). Standard negative reference material (USP, Rockville, Maryland) was the negative control. Three 60-mm tissue culture plates per dose were seeded with BALB/c-3T3 or CHO cells (70,000 cells/mL) to obtain test cultures with 350,000 cells per plate. After 24-h incubation, cultures were treated with CSC or positive controls, negative control, or the solvent DMSO. Cells were incubated for 48 h with the CSC or controls. CSCs were tested at 25, 50, 100, 150, or 200  $\mu\text{g}/\text{mL}$ . All treatments were performed in triplicate. After incubation, cells were washed with Dulbecco's Balanced Salt Solution (calcium and magnesium free) and re-fed with growth medium containing 0.05  $\mu\text{g}/\text{mL}$  neutral red dye. After 30 min, cultures were examined by inverted light microscopy for dye intake by live cells, cell lysis, intracytoplasmic granulation, and cell rounding (detachment). The magnitude of the cytotoxic responses was expressed by a descriptive rating of reactivity (none to severe) and grade (0–4) based on the condition of the cells. The technician graded the cells blindly and then decoded the label to get the concentration. A response was considered acceptable for evaluation if the replicate samples of the CSC did not differ by more than one grade. On two or more independent days one or more preliminary assay(s) were conducted to harmonize the assay conditions followed by a successful trial. A stratified RIDIT analysis based on the method of Fleiss was used to judge the equivalency of the ratings with the two cell types (Fleiss, 1986). Both assays were conducted in compliance with Good Laboratory Practice Standards.

## 3. Results

### 3.1. Multiple cytotoxicity endpoint assay

#### 3.1.1. Relative cell growth

With metabolic activation, BRI had the lowest EC<sub>50</sub> (113  $\mu\text{g}/\text{mL}$ ). EC<sub>50</sub>s for 2R4F (150  $\mu\text{g}/\text{mL}$ ) and CHAR (155  $\mu\text{g}/\text{mL}$ ) were similar. EC<sub>50</sub>s for LT (176  $\mu\text{g}/\text{mL}$ ) and LTMAS (173  $\mu\text{g}/\text{mL}$ ) were also similar. The highest measured EC<sub>50</sub> was BUR (187  $\mu\text{g}/\text{mL}$ ). Condensates FF and REC did not achieve a 50% reduction in cell growth at the tested concentrations. Calculated EC<sub>50</sub> values are presented in Table 2.

In the test system without metabolic activation, 2R4F had the lowest EC<sub>50</sub> (69  $\mu\text{g}/\text{mL}$ ), followed by CHAR (77  $\mu\text{g}/\text{mL}$ ), BRI (81  $\mu\text{g}/\text{mL}$ ), and BUR (99  $\mu\text{g}/\text{mL}$ ). LT showed an EC<sub>50</sub> near the highest concentration tested. LTMAS, FF, and REC did not achieve an EC<sub>50</sub> at the tested concentrations. Calculated EC<sub>50</sub> values are presented in Table 2.

**Table 2**  
Relative effective concentrations (EC50s) and rankings of CSCs<sup>a</sup>.

CSC <sup>b</sup>	TK-6 <sup>c</sup>		NHBE <sup>d</sup>	SAEC <sup>d</sup>	MVEC-L <sup>d</sup>
	With metabolic activation	Without metabolic activation			
2R4F	150 (2)	69 (1)	92 (2)	128 (2)	160 (5)
LT	176 (5)	209 (5)	99 (4)	296 (7)	125 (2)
LTMAS	173 (4)	212 (6)	117 (5)	135 (3)	158 (4)
FF	393 (8)	303 (7)	98 (3)	137 (4)	141 (3)
REC	280 (7)	343 (8)	132 (6)	357 (8)	309 (7)
BRI	113 (1)	81 (3)	15 (1)	46 (1)	32 (1)
BUR	187 (6)	99 (4)	317 (8)	202 (5)	295 (6)
CHAR	155 (3)	77 (2)	262 (7)	219 (6)	350 (8)

<sup>a</sup> All units are microgram per milliliter. The concentration for 50% cytotoxicity (EC50) was calculated from the dose–response equation derived by linear regression analysis of a plot of the logarithm of the cytotoxicity endpoint versus concentration.

<sup>b</sup> CSCs 2R4F, LT, FF, REC, BRI, BUR, and CHAR were produced under standard ISO smoking machine conditions; CSC LTMAS was produced under Massachusetts intense smoking machine conditions. CSC LT and LTMAS were generated from the same commercial US “light” non-menthol cigarette.

<sup>c</sup> Test concentration that reduced cell growth in human lymphoblastoid (TK-6) cells by 50% (EC50) based on cell growth (cell count) relative to the solvent control.

<sup>d</sup> Test concentration that reduced cell growth in human microvascular endothelial lung cells (HMVEC-L), normal human bronchial epithelial cells (NHBE), or human small airway epithelial cells (SAEC) by 50% (EC50) based on survival (MTT metabolism) relative to the solvent control.

### 3.1.2. Apoptosis and necrosis

With metabolic activation the frequency of apoptotic cells for all condensates was comparable to solvent controls. The frequency of necrotic cells was increased for FF at 200 µg/mL (13.7%), BUR at 150 µg/mL (50.1%) and 200 µg/mL (46.1%), and CHAR at 150 µg/mL (5.9%) and 200 µg/mL (22.6%) ( $p < 0.001$ ). Trend tests for FF, BUR, and CHAR showed a positive dose–response relationship for necrotic cells ( $p < 0.001$ ).

In the test system without metabolic activation, only condensate LTMAS produced an increase in the frequency of apoptotic cells at 200 µg/mL (13.3%) ( $p < 0.001$ ) relative to solvent controls. The frequency of necrotic cells was higher for FF at 150 µg/mL (2.1%) and 200 µg/mL (3%) and for LTMAS (17.3%) and REC at 200 µg/mL (22.6%) ( $p < 0.001$ ). Trend tests for LTMAS showed a positive dose–response relationship for apoptotic ( $p < 0.001$ ) and necrotic ( $p < 0.001$ ) cells. Trend tests for FF and REC showed a positive dose–response relationship for necrotic cells ( $p < 0.001$ ).

Fig. 1 illustrates the percentage of viable, apoptotic, and necrotic cells following treatments with 200 µg/mL of CSC.

### 3.2. Multiple cell-type co-culture system

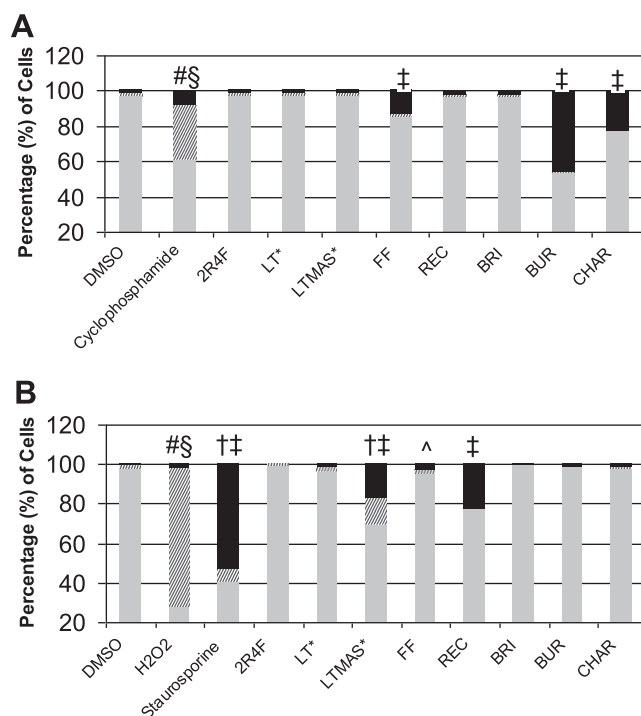
#### 3.2.1. MTT reduction

Human S-9 treatment led to significant cytotoxicity based on cell morphological observations in NHBE, SAEC, and MVEC-L cells exposed to CSCs (data not shown). The results were not used in data analysis.

Relative viability data are presented for NHBE, SAEC, and MVEC-L cells exposed to CSC in the absence of metabolic activation (Table 3). All CSCs reduced the viability of the cell lines. With some condensates, a stimulatory effect was seen at low concentrations. Low concentrations of 2R4F, LT, LTMAS, and FF had a stimulatory effect in SAEC as evidenced by increased cell viability. Low concentrations of 2R4F, LT, LTMAS, FF, REC, and CHAR increased cell viability in MVEC-L (Table 3).

In NHBE, excluding BRI, the reduction in viability ranged from 1.5-fold (BUR and CHAR) to 5.6-fold (2R4F). Condensate BRI was the most cytotoxic in NHBE cells. All test concentrations of BRI reduced viability to no more than 49% (Table 3). The EC50 for BRI was 15 µg/mL. The EC50s for 2R4F, LT, LTMAS, FF, and REC ranged from 92 to 132 µg/mL. The EC50s for condensates BUR and CHAR were more than 200 µg/mL (Table 2).

In SAEC, CSCs produced between a 1.3-fold (REC) and a 17-fold (2R4F) range in reduction in viability (Table 3). Condensate BRI was the most cytotoxic in SAEC cells with an EC50 of 46 µg/mL. Moderate cytotoxicity occurred with 2R4F, LTMAS, and FF (EC50 ranged



**Fig. 1.** Percentage of viable, apoptotic, or necrotic human lymphoblastoid TK-6 cells following treatment with cigarette smoke condensates (CSCs)\*. \*CSCs 2R4F, LT, FF, REC, BRI, BUR, and CHAR were produced under standard ISO smoking machine conditions; CSC LTMAS was produced under Massachusetts intense smoking machine conditions. CSC LT and LTMAS were generated from the same commercial US “light” non-menthol cigarette. The gray portion of the bar represents the percentage of viable cells, diagonal lines represent the percentage of apoptotic cells, and black represents the percentage of necrotic cells. Panel A: TK-6 cells exposed to dimethyl sulfoxide (DMSO), positive control, or CSCs for four hours in the presence of metabolic activation (S-9). Cyclophosphamide 30 µg/mL. CSCs 200 µg/mL. #, Apoptotic cells  $p < 0.001$  versus untreated culture. §, Necrotic cells  $p < 0.001$  versus untreated culture. †, Necrotic cells  $p < 0.001$  versus DMSO. Panel B: TK-6 cells exposed to DMSO, positive controls, or CSCs for 4 h without metabolic activation. H<sub>2</sub>O<sub>2</sub> = hydrogen peroxide 100 µM. Staurosporine 100 nM. CSCs 200 µg/mL. †, Apoptotic cells,  $p < 0.001$  versus DMSO; ‡, necrotic cells  $p < 0.001$  versus DMSO; #, apoptotic cells  $p < 0.001$  versus untreated culture; §, necrotic cells  $p < 0.001$  versus untreated culture; ^, necrotic cells  $p < 0.05$  versus DMSO. Comparisons between cell viability rates following treatment with solvent, control, or CSC were carried out using the Chi-square test.

from 128 to 170 µg/mL). The lowest cytotoxicity occurred with condensates LT, REC, BUR, and CHAR (EC50s greater than 200 µg/mL) (Table 2).

**Table 3**Relative viability of NHBE, SAEC, and MVEC after treatment with cigarette smoke condensates (CSCs).<sup>a</sup>

Concentration ( $\mu\text{g}/\text{mL}$ )	2R4F Mean (SEM) <sup>b</sup>	LT Mean (SEM)	LTMAS Mean (SEM)	FF Mean (SEM)	REC Mean (SEM)	BRI Mean (SEM)	BUR Mean (SEM)	CHAR Mean (SEM)
<i>NHBE</i>								
25	93.1 (4.8)	88.9 (6.3)	104.6 (6.3)	91.9 (6.4)	77.3 (5.0)	31.5 (3.3)	100.2 (14.3)	89.3 (1.7)
50	91.7 (4.2)	3.3 (3.4)	71.9 (10.4)	91.5 (6.3)	91.5 (5.0)	27.7 (2.6)	78.3 (6.6)	76.3 (2.5)
100	41.3 (4.4)	40.7 (3.3)	40.1 (6.2)	34.3 (0.4)	65.4 (4.9)	31.9 (3.7)	69.7 (2.9)	69.0 (2.8)
200	16.7 (2.2)	21.9 (1.3)	35.5 (10.8)	24.6 (7.1)	32.2 (2.1)	49.4 (6.4)	66.3 (0.7)	58.6 (0.8)
<i>SAEC</i>								
25	192.8 (0.9)	180.0 (5.9)	148.5 (11.3)	132.4 (2.9)	77.8 (11.0)	81.1 (4.6)	95.5 (6.0)	106.3 (6.8)
50	207.8 (2.6)	200.6 (2.9)	125.4 (6.8)	133.6 (4.8)	88.3 (2.9)	35.2 (2.8)	79.0 (2.8)	101.6 (5.5)
100	141.3 (7.5)	139.0 (10.5)	80.0 (8.0)	86.5 (1.6)	93.0 (19.3)	31.6 (16.3)	80.1 (1.8)	95.9 (9.6)
200	11.3 (5.8)	80.0 (4.3)	24.3 (2.9)	24.0 (1.3)	60.5 (1.8)	20.6 (3.6)	48.7 (0.3)	51.1 (2.7)
<i>MVEC-L</i>								
25	172.8 (5.1)	200.7 (7.3)	131.3 (4.8)	131.8 (10.5)	106.8 (3.0)	67.7 (8.8)	98.7 (4.8)	115.0 (6.5)
50	172.2 (3.2)	176.1 (3.7)	106.4 (8.8)	118.9 (4.0)	141.9 (6.2)	24.3 (3.4)	93.4 (5.3)	120.6 (10.5)
100	121.0 (8.3)	133.1 (21.3)	81.5 (6.2)	88.6 (3.5)	116.2 (0.9)	23.4 (2.9)	74.2 (0.8)	108.3 (5.5)
200	29.0 (3.4)	11.3 (5.8)	35.7 (1.1)	26.5 (3.3)	67.1 (1.5)	34.4 (3.3)	65.0 (0.5)	73.3 (1.7)

<sup>a</sup> CSCs 2R4F, LT, FF, REC, BRI, BUR, and CHAR were produced under standard ISO smoking machine conditions; CSC LTMAS was produced under Massachusetts intense smoking machine conditions. CSC LT and LTMAS were generated from the same commercial US "light" non-menthol cigarette. There were three replicates at each dose level.

<sup>b</sup> Absorbance of the formazan solution measured at 490 nm to determine the viability of the CSC treated human microvascular endothelial lung cells (HMVEC-L), normal human bronchial epithelial cells (NHBE), or human small airway epithelial cells (SAEC) relative to the DMSO solvent control  $\pm$  SEM.

The reduction in relative viability in MVEC across CSCs ranged from 1.5-fold (BUR) to 18-fold (LT) (Table 3). Condensate BRI was the most cytotoxic in MVEC-L cells with an EC<sub>50</sub> of 32  $\mu\text{g}/\text{mL}$ . The EC<sub>50</sub> of condensates 2R4F, LT, LTMAS, and FF ranged from 125 to 160  $\mu\text{g}/\text{mL}$ . The EC<sub>50</sub>s for condensates REC, BUR and CHAR were more than 200  $\mu\text{g}/\text{mL}$  (Table 2).

### 3.3. Neutral dye assay

#### 3.3.1. BALB/c-3T3 cells

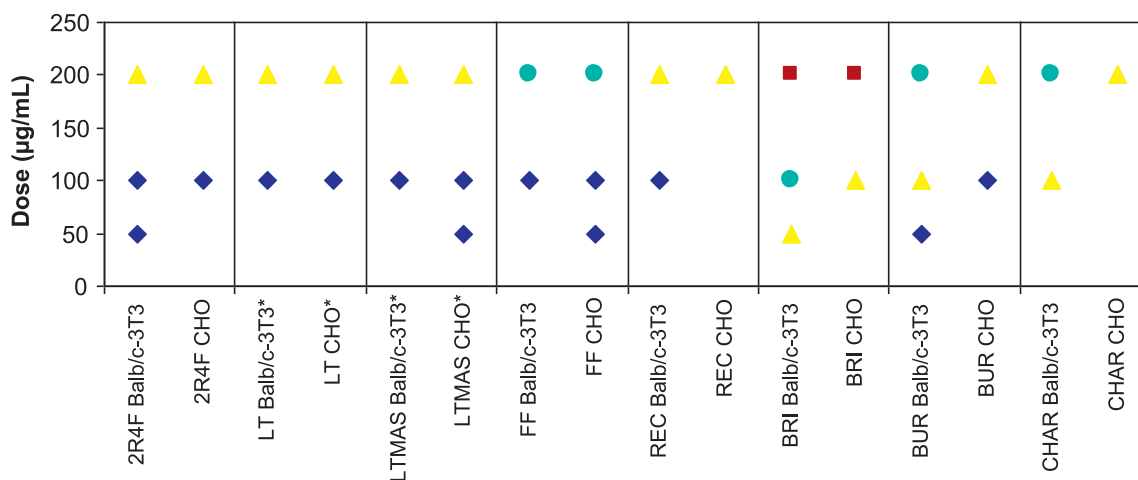
Condensate 2R4F was slightly (not more than 20% of cells rounded, loosely attached, and without intracytoplasmic granules, occasional lysed cells present) to mildly (not more than 50% of cells rounded and without intracytoplasmic granules, no extensive cell lysis and empty areas between cells) cytotoxic at 50  $\mu\text{g}/\text{mL}$  and above. Condensates LT, LTMAS, and REC showed slight and mild cytotoxicity at 100 and 200  $\mu\text{g}/\text{mL}$ , respectively. Condensate FF was slightly and moderately (not more than 70% of the cell layers

contain rounded cells or are lysed) cytotoxic at 100 and 200  $\mu\text{g}/\text{mL}$ , respectively. Condensate BUR showed slight to moderate cytotoxicity at 50  $\mu\text{g}/\text{mL}$  and above. Condensate CHAR produced mild and moderate cytotoxicity at 100 and 200  $\mu\text{g}/\text{mL}$ , respectively. Condensate BRI was the most cytotoxic, showing mild to severe (nearly complete destruction of the cell layers) cytotoxicity at 50  $\mu\text{g}/\text{mL}$  and above (Fig. 2).

#### 3.3.2. Chinese hamster ovary cells

Condensates 2R4F, LT, and BUR showed slight and mild cytotoxicity at 100 and 200  $\mu\text{g}/\text{mL}$ , respectively. Condensate LTMAS and FF were slightly toxic at 50 and 100  $\mu\text{g}/\text{mL}$  while LTMAS was mildly cytotoxic and FF was moderately cytotoxic at 200  $\mu\text{g}/\text{mL}$ . The highest test concentrations of REC and CHAR were only mildly cytotoxic. Condensate BRI was mildly cytotoxic at 100  $\mu\text{g}/\text{mL}$  and severely cytotoxic at 200  $\mu\text{g}/\text{mL}$  (Fig. 2).

In addition to a graphical presentation of the data (Fig. 2) a stratified RIDIT Fleiss analysis of the reactivity rating scores indi-



**Fig. 2.** Cytotoxicity of cigarette smoke condensates (CSCs) in Balb/c-3T3 or Chinese hamster ovary (CHO) cells in the neutral dye assay. \*CSCs 2R4F, LT, FF, REC, BRI, BUR, and CHAR were produced under standard ISO smoking machine conditions; CSC LTMAS was produced under Massachusetts intense smoking machine conditions. CSC LT and LTMAS were generated from the same commercial US "light" non-menthol cigarette. ♦, Slight reactivity; not more than 20% of cells rounded, loosely attached, and without intracytoplasmic granules, occasional lysed cells present. ▲, Mild reactivity; not more than 50% of cells rounded and without intracytoplasmic granules, no extensive cell lysis and empty areas between cells. ●, Moderate reactivity; not more than 70% of the cell layers contain rounded cells or are lysed. ■, Severe reactivity; near complete destruction of the cell layers.

cated that scores for the CSCs in CHO cells are significantly different from scores for the same CSCs in BALB/c-3T3 ( $p < 0.01$ ).

### 3.4. Comparisons across assays

Cytotoxicity rankings in the MEC assay.

With metabolic activation: BRI > BUR > CHAR > LTMAS > LT > 2R4F > FF > REC;

Without metabolic activation: BRI > BUR > CHAR > 2R4F > LTMAS > LT > FF > REC.

Cytotoxicity rankings in the MCTCC assay.

NHBE: BRI > 2R4F > FF > LT > LTMAS > REC > CHAR > BUR;  
SAEC: BRI > 2R4F > LTMAS > FF > BUR > CHAR > LT > REC;  
MVEC-L: BRI > LT > FF > LTMAS > 2R4F > BUR > REC > CHAR.

Cytotoxicity rankings in the NDA assay:

CHO cells: BRI > FF > LTMAS > 2R4F, LT and BUR > REC and CHAR;  
BALB/c-3T3 cells: BRI > BUR > CHAR > FF > 2R4F > LT, LTMAS and REC.

Across assays, BRI was consistently the most potent CSC tested. Other single-tobacco type CSCs were BUR and REC. REC was frequently the least potent CSC tested while BUR varied, depending on the assay. BUR was more cytotoxic in TK-6 cells and BALB/c-3T3 cells and less cytotoxic in human bronchial epithelial cells. LT and 2R4F have similar smoke, tar, and nicotine levels. These two CSCs had similar potency rankings in the MCE and NDA assays (rankings of 4th to 6th) but there was more spread in the rankings of these two CSCs across the primary human cell types of the MCTCC assay (rankings of 2nd to 7th).

### 3.5. Cytotoxic potencies based on nicotine levels

We and others have previously expressed CSC toxicity on a per-mg nicotine basis to compare toxicity across cigarettes with consideration of compensatory smoking behavior (DeMarini et al., 2008; Laugesen and Fowles, 2006; Rickert et al., 2007b). Similarly, in this study, the EC50 values were expressed on a per-mg nicotine basis as calculated from RCG per-mg nicotine (MCE) and viable cells per-mg nicotine (MCTCC) (Table 4). With or without metabolic activation, there was little change in the rank order of the

CSCs in the MCE assay compared to rank order of EC50s expressed per microgram of CSC. Two CSCs that changed rank order were BUR and 2R4F. Without metabolic activation, the EC50 of BUR decreased and the EC50 of 2R4F increased when expressed on a per nicotine basis.

Interestingly, BRI was the most cytotoxic in the MCTCC assay when expressed per microgram of CSC. However, when expressed per-mg nicotine, LT and a cigarette blended to be representative of a US low-tar cigarette, 2R4F, were the most cytotoxic CSCs in NHBE and MVEC-L cells and 2R4F in SAEC cells. Overall, REC most frequently had the highest EC50 and BRI the lowest EC50, regardless of the assay or cell type.

Expressed per microgram of CSC, the EC50 values in the MCE had a 3.5-fold range across CSCs with metabolic activation and a 5-fold range without metabolic activation. In the MCTCC, there was a much wider range in EC50 values across CSCs ranging from 8-fold for SAEC cells to 11-fold in MVEC-L cells to 21-fold in NHBE cells.

In contrast, when expressed on a per-mg nicotine basis, there was a narrower range in EC50 values across the CSCs (MCE: 1.7-fold with metabolic activation and 1.6-fold without; and MCTCC: NHBE 1.8-fold, MVEC-L 1.8-fold and SAEC 2.4-fold).

## 4. Discussion

The cytotoxicity of cigarette smoke has been reported in the published literature (Andreoli et al., 2003; Bombick et al., 1997a,b; Pouli et al., 2003). This study addresses a variety of CSCs in multiple assays with endpoints that represent different cellular responses to a toxic exposure. In the MCE assay, CSCs reduced cellular growth in TK-6 cells with or without metabolic activation. In the presence of metabolic activation, CSC from cigarettes composed of 100% flue-cured tobacco (BRI) had the lowest EC50 (113  $\mu\text{g}/\text{mL}$ ). The highest EC50 was from the CSC of a commercial full-flavored cigarette (FF) (393  $\mu\text{g}/\text{mL}$ ). The nicotine content of BRI CSC was 2 $\times$  higher than the nicotine content of FF CSC (2.34 mg/mL and 0.98 mg/mL, respectively). When results were expressed on a per-mg of nicotine basis, BRI was still the most toxic, and FF remained among the least potent, suggesting that chemicals other than nicotine influence the toxicity of these CSCs. Bright (flue-cured) tobacco has high levels of several chemicals including sugars, amino acids, and polyphenols (University of Kentucky 1993 <http://www.bae.uky.edu/Publications/AEUs/aeu-93.pdf>). Phenolic compounds (hydroquinones, catechols) generated when polyphenolic compounds in tobacco are burned are cytotoxic and are thought to contribute to the oxidative stress caused by tobacco

**Table 4**  
Relative effective concentrations (EC50s) and potency rankings of cigarette smoke condensates (CSCs) expressed per milligram of nicotine.<sup>a</sup>

CSC <sup>b</sup>	TK-6 <sup>c</sup>		NHBE <sup>d</sup>	SAEC <sup>d</sup>	MVEC-L <sup>d</sup>
	With metabolic activation	Without metabolic activation			
2R4F	502 (6)	400 (4)	360(1)	247 (1)	390 (2)
LT	461 (5)	517 (6)	374 (2)	497(7)	310 (1)
LTMAS	459 (4)	508 (5)	427 (4)	369 (3)	417 (4)
FF	562 (7)	545 (7)	390 (3)	380 (4)	392 (3)
REC	564 (8)	591 (8)	471 (5)	604 (8)	564 (8)
BRI	330 (1)	373 (1)	638 (8)	351 (2)	434(5)
BUR	414 (2)	379 (2)	495 (6)	447 (5)	485 (6)
CHAR	455 (3)	390 (3)	540 (7)	491 (6)	546 (7)

<sup>a</sup> The concentration for 50% cytotoxicity (EC50) was calculated from the dose–response equation derived by linear regression analysis of a plot of the logarithm of the cytotoxicity endpoint versus concentration.

<sup>b</sup> CSCs 2R4F, LT, FF, REC, BRI, BUR, and CHAR were produced under standard ISO smoking machine conditions; CSC LTMAS was produced under Massachusetts intense smoking machine conditions. CSC LT and LTMAS were generated from the same commercial US “light” non-menthol cigarette.

<sup>c</sup> Test concentration that reduced cell growth in human lymphoblastoid (TK-6) cells (TK6 tester strain: CRL-8015 (Source-ATCC, Manassas, VA)) by 50% (EC50) based on cell growth (cell count) relative to the solvent control ( $n = 3$ ). Units are relative cell growth per-mg nicotine.

<sup>d</sup> Test concentration that reduced cell growth in human microvascular endothelial lung cells (HMVEC-L), normal human bronchial epithelial cells (NHBE), or human small airway epithelial cells (SAEC) by 50% (EC50) based on survival (MTT metabolism) relative to the solvent control. Units are viable cells per-mg nicotine.

smoke exposure (Wooten et al., 2006). The particulate matter of bright tobacco has higher levels of hydroquinones and catechol than burley tobacco (Wooten et al., 2006) and thus these compounds may contribute to the higher toxicity of BRI CSC. The order of CSC EC50s was essentially unchanged when the MCE assay was conducted without metabolic activation. However, some EC50 levels were approximately 2-fold lower (2R4F, BUR, CHAR), suggesting that enzymatic metabolism inactivates some cytotoxic chemicals in these CSCs.

The EC50 rank of only a few CSCs changed more than one position in the MCE assay when expressed on a per-mg nicotine basis. With metabolic activation, 2R4F moved down from second to sixth and BUR moved up from sixth to second. Without metabolic activation, 2R4F moved down from first to fourth, BRI moved up from third to first, and BUR moved up from fourth to second. REC and FF were consistently the two least toxic CSCs in the presence or absence of metabolic activation in the MCE assay when expressed on a condensate basis or a nicotine basis. These two cigarettes had similar tar and nicotine smoke levels, while the nicotine content of FF CSC is approximately 50% higher than that of REC.

Apoptosis and necrosis are involved in the terminal events leading to the death of the cell and may provide complementary information to cellular growth rates. Apoptosis is characterized by distinct morphological characteristics and it occurs in a number of scenarios, including chemical-induced cell death (Elmore, 2007). Necrotic cells indicate cell death and the subsequent post-mortem changes and can result from severe disturbance in cellular metabolism (Kanduc et al., 2002). Cigarette smoke-induced cellular proliferation, apoptosis, and necrosis are associated with oxidative stress in a dose-dependent manner (Hoshino et al., 2001; Luppi et al., 2005). Reactive cigarette smoke constituents such as acrolein are thought to produce inflammatory conditions through apoptotic and necrotic mechanisms although the contribution of stable and cell membrane-permeable free radicals such as hydroquinone cannot be discounted (Aoshiba et al., 2001; Hoshino et al., 2001; Li et al., 1997; Misonou et al., 2006; Ramage et al., 2006). Others have suggested that the nicotine content of CSC attenuates apoptosis (Chen et al., 2008). We did not observe concordance between cell growth and the presence of apoptotic or necrotic cells. An increased frequency of apoptotic human lymphoblastoid TK-6 cells was produced only by the highest dose of LTMAS (-S9), a CSC with an EC50 ranked 6th on a condensate basis and 5th on a milligram nicotine basis. The nicotine content of LTMAS (1.16 mg/mL) was higher than that of other CSCs such as REC (0.64 mg/mL) that did not produce an increase in apoptotic cells, again suggesting that other factors, possibly owing to intense smoking conditions, play a substantial role in the oxidative stress and cell death caused by CSC. Relative yields of several compounds, including phenol and cresols, are increased to a greater extent than other smoke constituents when the filter vents of higher ventilation cigarettes were blocked as was done with LTMAS (Counts et al., 2005). Use of other cell types or multiple time points to examine cells for apoptotic changes may produce a different pattern of response. The percentage of necrotic LTMAS-treated cells was also significantly increased in the absence of metabolic activation. No increase in apoptotic or necrotic cells was seen with CSC from the same cigarette smoked under less intense machine conditions (LT) with similar EC50 rankings. We previously reported a similar pattern in a separate study where the same LTMAS CSC was more mutagenic in *Salmonella* than the LT CSC (DeMarini et al., 2008). Others have reported that mainstream smoke condensate produced under intense smoking machine conditions is less cytotoxic and mutagenic than condensate produced under less intense conditions (Rickert et al., 2007b; Roemer et al., 2009).

In addition to LTMAS, several other CSCs increased the frequency of necrotic TK-6 cells. However, FF (among the least potent of the CSCs in terms of cellular growth) was the only condensate to

increase the frequency of necrotic cells both with and without metabolic activation, suggesting that this CSC contains compounds that are cytotoxic as the parent compound and others that are metabolized to a cytotoxic form. Both endpoints (apoptosis/necrosis and relative cellular growth) were positive within the range of concentrations tested for BUR and CHAR (+S9) and LTMAS.

The results from the IdMOC MCTCC assay showed that a diverse set of CSCs can be classified into three groups based on toxicity to human pulmonary cell types. BRI represents the most cytotoxic group, with EC50s that ranged from 15 to 46  $\mu\text{g}/\text{mL}$  for the three cell types. 2R4F, LT, LTMAS, and FF displayed intermediate cytotoxicity, with EC50s between 92 and 296  $\mu\text{g}/\text{mL}$ . REC, BUR, and CHAR demonstrated the least toxicity in this assay, with BUR and CHAR having EC50 values consistently greater than the highest concentration evaluated of 200  $\mu\text{g}/\text{mL}$  for all three cell types. Cytotoxicity results did not show great differences amongst the three cell types tested. However, NHBE appears to be more sensitive to the CSCs, as illustrated by the relatively lower EC50 values than in the other two cell types. Stimulatory effects (increased cell count) were clearly noted for CSCs with intermediate cytotoxicity (2R4F, LT, LTMAS, and FF) in SAEC and MVEC, suggesting an increase in MTT cleavage presumably due to oxidative stress after the 24-h treatment period. In contrast, no stimulatory effect was observed in NHBE for any of the condensates.

The stimulatory effects seen in two of the three cell lines observed in the IdMOC MCTCC assay are consistent with the known increased airway epithelial proliferation in smokers (Sekhon et al., 1994). Luppi reported that low concentrations of CSC increased proliferation of cultured human carcinoma lung cells from a bronchial epithelial cell line, while high concentrations were cytotoxic (Luppi et al., 2005). Our study is the first report of stimulatory effects of cigarette smoke condensate in normal human airway epithelial cells and in microvascular endothelial cells.

In the NDA assay with CHO cells, FF and BRI were most cytotoxic showing moderate and severe cytotoxicity, respectively, at 200  $\mu\text{g}/\text{mL}$ . BALB/c-3T3 cells appeared more sensitive to CSC cytotoxicity than did CHO cells. In BALB/c-3T3 cells, BRI again produced the most severe cytotoxicity, showing mild to severe cytotoxicity at 50  $\mu\text{g}/\text{mL}$  and higher. At the highest concentrations tested, FF, BUR, and CHAR were moderately cytotoxic.

2R4F, LT, LTMAS, and REC failed to produce more than mild cytotoxicity (not more than 50% of cells round and without intracytoplasmic granules) and failed to produce cytotoxicity grades greater than 2 in either cell type at the dose levels tested.

An objective of this study was to distinguish between viable, damaged, and dead cells in cells with different levels of metabolic activity, using the morphological viability component of the neutral dye assay. Balb/c-3T3 clone A31 cells are metabolically active (Lubet et al., 1990) while CHO cells have an insignificant level of xenobiotic metabolic activity (Teepe et al., 1992). Both CHO and Balb/3T3 cells have been used by others in the spectrophotometric component of the neutral dye exclusion assay (Bombick et al., 1997b, 1998; Borenfreund and Puerner, 1985; Putnam et al., 2002; Roemer et al., 2009). Analysis of the results from this study suggests that these cell types do not produce equivalent responses ( $p < 0.01$ ) with equivalent doses of CSC and suggest that cell type can influence the outcome of a CSC cytotoxicity study. The influence of metabolism in the assessment of CSC cytotoxicity is further suggested by the apparent overlap in results of the NDA and MCE. Four CSCs producing no more than mild toxicity in metabolically active Balb/c-3T3 cells (2R4F, LT, LTMAS, and REC) similarly did not show an increase in apoptosis or necrosis in TK-6 cells with S9 while two CSCs that produced moderate toxicity (FF and BUR) in the NDA assay had a significant increase in necrotic cells. A similar pattern was seen when comparing the results in TK-6 cells without metabolic activation and NDA results in CHO cells.

Others have reported that the toxicity of CSC without S9 is higher than the toxicity of CSC with rat liver S9 in an in vitro assay using normal human peripheral blood cells (Jianlin et al., 2009). Although we observed significant cytotoxicity in human cells exposed to CSC and human S9 in the MCTCC assay, we found some CSC EC50s to be higher (2R4F, FF, BRI, BUR, CHAR) and some to be lower (LT, LTMAS, REC) in the presence of rat liver S-9 in the MCE assay than without S-9. The mixed results may be explained in part by differences in the chemical composition of the CSC owing to the blend of additives, tobaccos, and tobacco-derived materials used to manufacture the cigarette (e.g., “light” cigarettes such as that used to produce CSCs LT and LTMAS may contain a higher percentage of reconstituted tobacco (REC) than higher tar cigarettes (Hoffmann and Hoffmann, 1996)) and also by differences in the endogenous metabolic capabilities of various primary and cultured cells types.

Charcoal filters selectively remove gas phase chemicals from cigarette smoke (Polzin et al., 2008; Smith et al., 2006). It would not be expected that the selective filtration of gas phase chemicals would affect the toxicity of CHAR condensate as it consists of particulate phase smoke constituents (Bombick et al., 1997a). CSC from a charcoal filtered cigarette smoke would be expected to have potency rankings similar to or higher than that of a CSC with a standard cellulose acetate filter and the same smoke tar level (e.g., FF in this study) because the charcoal reduces the amount of cellulose acetate available to filter out particulate phase chemicals (Hoffmann et al., 1996). Although results in Balb/c-3T3 cells in the NDA assay are consistent with this hypothesis, potency rankings for CHAR and FF in the MCE and MCTCC appear to be in opposition, both between assays and between CSCs.

Curvall and colleagues fractionated cigarette smoke condensate from nonfilter American blend cigarettes. The greatest cytotoxic activity resided in the neutral fraction which they found to contain oxygenated terpenoids, alkylindoles, cyclopentenones, oxygenated isoprenoids, and compounds containing an alpha, beta-unsaturated carbonyl moiety (Curvall et al., 1985). Findings in this study suggest that hydroquinone and catechol and their derivatives may contribute substantially to the cytotoxicity of CSC. Additional research is needed to assess the effect of reductions in these compounds on the cytotoxicity of cigarette smoke in these short-term in vitro assays.

We observed that flue-cured tobacco smoke condensate is highly cytotoxic, a finding supported by others (Bombick et al., 1998). The greater toxicity of flue-cured tobacco suggests that, following confirmatory research, certain chemicals are potential targets for removal in efforts to reduce the toxicity of the particulate phase of cigarette smoke. The nicotine content of the CSCs failed to explain the pattern of toxicity seen in the assays.

LT and LTMAS had similar cytotoxicity rankings in some, but not all, assays. As noted above, LTMAS, but not LT, produced a significant increase in apoptotic and necrotic TK-6 cells in the MCE assay. In normal human respiratory cells, LTMAS and LT were ranked 3rd and 7th, respectively, in small airway cells when expressed on either a condensate or nicotine basis. The hypothesis that smokers consume a cigarette to attain a desired quantity of nicotine (Warner and Slade, 1992) raises concerns as “light” cigarettes (i.e., 2R4F and LT) in this study were the most potent in NHBE, MVEC, and SAEC (2R4F) cells when expressed on a per nicotine basis (MCTCC) or when generated under intense smoking machine conditions (MCE). And given that the leading lung cancer of smokers, adenocarcinoma, arises predominantly in small airway epithelial cells (Schueller et al., 2008), we have further support for the hypothesis that compensatory smoking behavior substantially raises the health risks from low-tar cigarettes.

In conclusion, these findings suggest that choice of cell type, presence or absence of metabolic activation, and smoking machine

conditions can influence the overall and relative toxicity ranking of cigarette smoke condensate in in vitro cytotoxicity assays. Results suggest that some tobacco types (flue-cured tobacco) and possibly yield category can increase the toxicity of smoke condensate, notably to human respiratory tract cells. Additional research is warranted to determine whether changes in smoke yields of candidate cytotoxic smoke constituents reduce the cytotoxicity of cigarette smoke without commensurate increases in genotoxicity or other measures of toxicity.

## 5. Conflict of interest statement

None of the authors report a conflict of interest.

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