CEBP Focus: Tobacco Research

Review

Evaluation of *In vitro* Assays for Assessing the Toxicity of Cigarette Smoke and Smokeless Tobacco

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Abstract

Background: ***In vitro* toxicology studies of tobacco and tobacco smoke have been used to understand why tobacco use causes cancer and to assess the toxicologic impact of tobacco product design changes. The need for toxicology studies has been heightened given the Food and Drug Administration's newly granted authority over tobacco products for mandating tobacco product performance standards and evaluate manufacturers' health claims about modified tobacco products. The goal of this review is to critically evaluate *in vitro* toxicology methods related to cancer for assessing tobacco products and to identify related research gaps.**
Methods: **PubMed database searches were used to identify tobacco-related *in vitro* toxicology studies published since 1980. Articles published before 1980 with high relevance also were identified. The data were compiled to examine (*a*) the goals of the study, (*b*) the methods for collecting test substances, (*c*) experimental designs, (*d*) toxicologic end points, and (*e*) relevance to cancer risk.**
Results: **A variety of *in vitro* assays are available to assess tobacco smoke that address different modes of action, mostly using non–human cell models. However, smokeless tobacco products perform poorly in these assays. Although reliable as a screening tool for qualitative assessments, the available *in vitro* assays have been poorly validated for quantitative comparisons of different tobacco products. Assay batteries have not been developed, although they exist for nontobacco assessments. Extrapolating data from *in vitro* studies to human risks remains hypothetical.**
Conclusions: ***In vitro* toxicology methods are useful for screening toxicity, but better methods are needed for today's context of regulation and evaluation of health claims.** (Cancer Epidemiol Biomarkers Prev 2009; 18(12):3263–304)

Introduction

Tobacco is smoked and used orally by people worldwide, and currently there are over 1.3 billion smokers (1, 2). Burning tobacco results in combustion, pyrolysis, and other chemical reactions that cause the smoker to be exposed to thousands of chemicals (1, 3-5). The use of smokeless tobacco (ST) also results in exposure to numerous chemicals and carcinogens, although less than for smoking. The attendant health consequences of using tobacco products are numerous, including cancer and diseases of the cardiovascular and respiratory systems (1, 6). In June 2009, the Food and Drug Administration has been granted legislative authority over all tobacco products. They are now enabled to mandate tobacco product performance standards and regulate advertising and packaging. The Food and Drug Administration also must evaluate health claims made by manufacturers in the context of reduced health risks, and this is done in the context of an Institute of Medicine study concluding that a feasible harm reduction strategy for smokers who will not or cannot quit, albeit not proven, to reduce smoke exposure through the use of modified tobacco products (7). Every major tobacco manufacturer has introduced these so-called potential reduced exposure products (PREP) into the marketplace over the last several years. The Institute of Medicine, the World Health Organization Study Group on Tobacco Product Regulation and others have recognized that although there may be opportunities to reduce smoking-related harm, there are also risks to adopting harm reduction strategies (8-18). However, human studies supporting the use of exposure reduction to reduce tobacco-related harm from cigarettes are almost nonexistent, although toxicology studies have been used for such purposes with numerous limitations. A similar harm reduction strategy is ongoing for ST as well. Specifically, in the United States, major cigarette tobacco manufacturers have begun to market ST modeled after Swedish snuff (snus), which are low tobacco-specific nitrosamine (TSNA) products. Importantly, the actual impact of PREPs on human health would need to be assessed in epidemiologic studies and clinical trials. However,

Received 9/17/09; accepted 10/7/09; published online 12/3/09.
Grant support: National Cancer Institute N01-PC-64402 Laboratory Assessment of Tobacco Use Behavior and Exposure to Toxins Among Users of New Tobacco Products Promoted to Reduce Harm.
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doi:10.1158/1055-9965.EPI-09-0965

before human use, laboratory tests with cell culture models can be used to screen products for changes in toxicity in the context of relative safety assessments.

The mechanisms by which tobacco smoke cause cancer and other tobacco-related diseases have been studied intensively during the past decades. Much has been learned through the use of toxicology methods, particularly experimental *in vitro* (cell culture) and *in vivo* (animal) studies. Compared with experimental animals studies, assays based on *in vitro* systems can be conducted quickly, are relatively inexpensive, and allow for the rapid screening of many samples (19, 20). *In vitro* assays are relatively easy to customize for specific research questions, e.g., elucidating cell-specific effects (21-23). Over the years, a panoply of tests have been used to asses tobacco toxicants; however, the interpretation of the data generated is not trivial for any tobacco product and particularly if the goal is to compare modified products. Almost all of the available *in vitro* toxicology methods (*a*) were not developed for testing tobacco and tobacco smoke toxicity, (*b*) are not reliably quantitative to allow valid comparisons of substantially different tobacco products with differing yields of complex chemical mixtures, (*c*) provide data that cannot reliably be extrapolated to infer human cancer risk, and (*d*) were intended primarily as screening methods for chemicals to identify possible humans carcinogens. Thus, existing methods need to be evaluated and validated, and new ones developed, to address these issues related to tobacco products.

The purpose of this article is to review the current state of the science on a compendium of *in vitro* toxicology methods for cancer pathways, and provide guidance about how they should be interpreted. This review will provide a comprehensive survey of *in vitro* toxicology methods that have been or could be applied to the testing of tobacco products. It will identify those assays that have been used for tobacco-related applications, identify the strengths and limitations of these methods and how they can be used together to assess tobacco products, and place these assays in the context of human risk assessment. The review will begin with discussing methods used for collecting tobacco smoke and ST extract for analysis, followed by a critical review of available methods for *in vitro* toxicology divided into sections on cytotoxicity, cell proliferation, cell cycle control, apoptosis, and genotoxicity assays. There also is a discussion on emerging technologies for *in vitro* toxicology assays. Last, the results will include choices of cell culture type and culture conditions, and the differing toxicology effects using different smoking machine regimens. The Discussion includes considerations for extrapolating *in vitro* results to human risk, the development of batteries of testing, criteria for validating assays, and research gaps. Several recent publications have reviewed *in vitro* toxicology for tobacco smoke and ST (24-26). They do not, however, critique the methods and/or identify research gaps in the context of comparing tobacco products and regulation. Unrelated to tobacco toxicology, over the last several years, the Expert Working Group on Hazard Identification has published several reports that are useful in understanding the uses and limitations of *in vitro* testing, which can be applicable to tobacco (27-29).

Materials and Methods

Tobacco-related toxicology methods and studies were identified through PubMed searches using the search terms: cigarette smoke or smokeless tobacco, and keywords related to the topic for each section, such as Ames, Salmonella, cell cycle assay, etc. Searches were limited to *in vitro* assays and to those published in the English language. All studies identified that were published after 1980 were reviewed, and citation lists within those articles were reviewed to ensure that the most complete list of publications have been identified. Articles published before 1980 with high relevance to the study of PREPs or low-yield cigarettes also were identified and reviewed. Studies to be cited in this review were selected based on whether or not they assessed a PREP or low-yield cigarettes, or ST products. The data were compiled to examine (*a*) the goals of the study, (*b*) the methods for collecting test substances from tobacco and tobacco smoke, (*c*) the experimental designs that were used, (*d*) the toxicologic end points, and (*d*) the relevance to human disease risk.

Results

Collecting Tobacco Smoke for Toxicology Testing. A prerequisite to the study of cigarette smoke toxicity is to generate and collect the smoke for testing. Over the years, this necessity has lead to the development of smoking machines of varying sophistication that use different puffing protocols and a plethora of different approaches by which to collect the smoke. In spite of several attempts over the last 80 years to develop standards for smoke extract generation, there is still much discussion about the most appropriate and best approaches, and the relevance of the materials generated to what smokers are really exposed to.

Analytic smoking machines were initially developed in the 1930s, when Pfyl et al. (30) observed that the smoking process influences the amount of tobacco smoke aerosols (Pfyl, 1933, cited in ref. 30). Since then, smoking machines have undergone many modifications and improvements. Commercially available smoking machines today are designed to accommodate different puff parameters and vary in the number of cigarettes that can be smoked concurrently or consecutively. Different designs are typically used for different purposes. Rotary machines smoke cigarettes consecutively and are ideally suited to smoking large numbers of cigarettes per unit time to generate large amounts of smoke for studies. In-line machines smoke cigarettes concurrently, are better suited for replicate analysis, and provide more flexibility for the testing of smoking regimens that better mimic more human-like puffing profiles.

As early as 1936, Bradford et al. (31), who worked for the American Tobacco Company, described the need for standardized smoking parameters that would aid in the characterization and reproducibility of cigarette smoke experiments in the laboratory. It was not until 1967 that the Federal Trade Commission (FTC) modified Bradford's protocol and adopted it as the U.S. standard.[3] A summary of the commonly used machine smoking parameters used

[3] http://legacy.library.ucsf.edu/tid/jgx60a99

Table 1. Smoking protocols for smoking machine

	FTC*	MDPH (271)	HC†
Puff volume (mL)	35	45	55
Puff duration (s)	2	2	2
Mean flow rate (mL/s)	17.5	22.5	27.5
Interpuff interval (s)	60	30	30
Ventilation holes (where applicable)	100%	50%	0 (all holes blocked)
Air flow (mm/s)	Not specified	Not specified	200 ± 50
Butt length	23 mm for nonfiltered cigarettes or the length of the filter overwrap plus 3 mm for filtered cigarettes	23 mm for nonfiltered cigarettes or the length of the filter overwrap plus 3 mm for filtered cigarettes	23 mm for nonfiltered cigarettes or the length of the filter overwrap plus 3 mm for filtered cigarettes

*http://legacy.library.ucsf.edu/tid/jgx60a99.
†http://www.hs-sc.gc.ca/hc-ps/tobac-tabac/legislation/reg/indust/method/_main-principal/nicotine-eng.php.

today are shown in Table 1. The FTC protocol has been the most widely used puffing regimen since then, specifying puffs of 35 mL of a 2-second duration and happening every 60 seconds, until the length of the cigarette butt is no less than 23 mm for nonfiltered cigarettes or the filter overwrap plus 3 mm for filtered cigarettes (30). At that time, it was considered that tar yields were meaningful in terms of exposure and health risk, and there needed to be a way for the smoker to compare one product to another. The choice of this puff profile was, however, arbitrarily determined, being based on personal observations, rather than being determined experimentally (30). Over time, modifications to cigarettes to reduce tar yields, such as filter ventilation and other novel designs, have rendered the smoking machine and the FTC method less relevant, or not relevant at all, to the estimation of human smoking exposure (30, 32). These modifications have influenced how a smoker will smoke their cigarettes and so any particular smoking machine protocol does not capture this effect, for example: current smokers puff cigarettes of lower tar yield typically at higher puff volumes and shorter intervals, and in some cases block filter ventilation holes (33, 34). Given the relevance issues of the FTC method to human smoking behavior, the FTC recently rescinded its guidance for using the FTC puffing methods.[4] Thus, there is no current specified smoking machine method used in the United States.

Outside the United States, specifications for the performance and use of smoking machines also have been developed by organizations such as the International Organization for Standardization (ISO)[5] and Cooperation Centre for Scientific Research Relative to Tobacco (CORESTA).[6] In addition to the puffing regimen, ISO also set standard conditions for physical components of the smoking machine: the cigarette holders, smoke traps, ports, channels, and ashtray specifications, as well as standard conditions for draw resistance, pressure drop, and compensation. The ISO smoking method uses the same puffing profiles as the FTC method.

Several attempts have been made to develop smoking profiles with more relevance to how cigarettes are smoked in the real world, such as those developed by the Massachusetts Department of Public Health (MDPH) and Health Canada (HC). These profiles increase the puff volumes to 45 and 55, respectively, keep the 2-second duration, but reduce the time between each puff to 30 seconds. The MDPH method blocks 50% of the filter ventilation holes, whereas the HC method blocks 100% of the holes. Importantly, how to mimic actual human smoking behavior on a smoking machine has received little attention, although some attempts had been made (33-36). For regulatory purposes, The WHO TobReg recommends using both ISO and HC machine smoking methods to obtain the range of toxicant deliveries under extreme conditions (37, 38). The application of smoking machine studies to mimic human smoking behavior has recently been reviewed (39).

After the smoke is generated by puffing, it can be collected for analysis in various ways, e.g., on a filter pad, in a cold trap, or as whole smoke (WS; see below). Over the years, a bewildering range of methods have been used for collecting smoke for toxicology studies, and the materials generated have been labeled in different nonstandard ways in the scientific literature. Table 2 summarizes the terminology that is most commonly used to describe the materials generated, and although some authors use the terms interchangeably, for the purposes of this review, we will adopt the definitions shown in Table 2. Most commonly, the terms total particulate matter (TPM) and cigarette smoke condensate (CSC) are used, and these terms are sometimes used synonymously. TPM is typically collected on a Cambridge filter pads, which are glass fiber filters required to retain at least 99.9% of all particles having a diameter of $\geq$0.3 μm of a dioctyl phthalate aerosol at a linear air velocity of 140 mm s^{-1} (40).[7] Smoke is drawn through the filter pad by the smoking machine pump controlling the puffs. TPM is typically eluted off the pad using DMSO. The Cambridge filter method has been used to collect TPM for a variety of *in vitro* toxicologic assays including cell cycle analysis, cytotoxicity, sister chromatid exchanges (SCE), chromosomal aberrations (CA), and Ames testing (21, 41-62).

CSC is collected by drawing the smoke through cold traps, as originally developed by Elmenhorst (63), and the cold trap remains the most common and practical method for collecting large quantities of smoke condensate.

[4] http://www.ftc.gov/opa/2008/11/cigarettetesting.shtm
[5] http://www.iso.org
[6] http://www.coresta.org
[7] http://www.coresta.org/Recommended_Methods/CRM_22.pdf

Table 2. Smoking machine collection method terminology

Name	Abbreviation	Explanation
Total particulate matter or wet total particulate matter	TPM or WTPM	Smoke collected on a Cambridge Filter pad that includes water and nicotine. TPM will generally not include volatile compounds that are in cigarette smoke. In the literature, TPM is sometimes called CSC.
Tar or nicotine-free dry particulate matter	Tar or NFDPM	Smoke collected on a Cambridge Filter pad that is the weight difference for TPM minus the nicotine and water (determined analytically, often by gas chromatography). In the literature, tar is sometimes called CSC.
Cigarette smoke condensate	CSC	Smoke fractions that are collected as a condensate, typically in a cold trap, but sometimes in impaction traps and by electrostatic precipitation. This method will collect some semivolatiles, but CSC will not contain volatile compounds. CSC is sometimes used interchangeably with TPM or tar.
Gas/vapor phase	GVP	Smoke emissions collected that passes through a Cambridge filter pad or cold trap.
Whole smoke	WS	Smoke is not collected but used to directly expose cells, e.g., the smoke is bubbled through media in a flask.

The traps, which often contain glass beads, are typically cooled in a dry ice-methanol mixture (−78°C). The low temperatures cause the smoke and ice particles to form a mat at the bottom of the trap. Large traps allow extracts to be prepared from as many as 5,000 cigarettes, as long as the traps are kept cold (64). Cold traps have been used for collection of CSC for *in vitro* toxicologic assays (65, 66) and for *in vivo* testing (e.g., for use in skin painting studies; refs. 67-70). Another design is to have traps that contain some type of liquid to dissolve smoke constituents. The liquids used for the trap may be PBS (71-73), culture media (74, 75), or acetone (76). The CSC collected from the trap is either aliquoted and frozen, or concentrated by heat evaporation, a stream of nitrogen gas, or by vacuum. The residual condensate is then dissolved in DMSO and stored until use (76, 77). Another method for collecting CSC is to use electrostatic precipitation (64), which uses a positive central electrode surrounded by a cylindrical negative electrode. The positive electrode produces an electric field that charges the smoke aerosol particles that are then collected at the negative electrode. Electrostatic precipitation is most commonly used to collect CSC for fractionation and trace metal studies (78-80), because glass and quartz filter pads contain these trace impurities (81). It should be noted that CSC and TPM do not contain all the chemical constituents from smoke, as various gases and volatile organic compounds pass through these collection systems. Electrostatic precipitation devices are claimed to have better collection efficiency because they are less flow and load dependent than Cambridge filter pads. Cold traps, however, offer several advantages over filter pad and electrostatic collection methods, including collecting from both the vapor and particulate phases, and that no high voltage electricity is used in the collection process that might impact condensate chemistry (64).

Tobacco smoke also can be assayed directly as WS into an assay vessel, or the constituents of smoke that pass through the Cambridge filter pad can be assayed as the gas/vapor phase (GVP). WS, when used directly into the assay vessel, is diluted with filtered and humidified air injected through an exposure control system into a containment chamber where cells are exposed at the air liquid interface (59, 82-87). This has the disadvantage of diluting the toxic effects of the smoke. Different WS exposure systems from several laboratories were assessed by the Cooperation Centre for Scientific Research Relative to Tobacco *In vitro* Toxicology Task Force who found remarkably similar results.[8] GVP is collected by a cold trap *in vitro* analyses (71, 74, 88, 89). For GVP experiments, a similar apparatus is used but the smoke that passes first through a filter pad is used (83, 90-94). The GVP also can be directly used in cell culture systems.

Standardizing the reporting of results for cigarette smoke toxicology studies can be done in different ways and typically it is expressed per unit of weight (e.g., per milligram of tar, TPM, or CSC) or per cigarette. The per cigarette basis is derived mathematically from the per milligram basis, e.g., the results per milligram of tar multiplied by the tar yield from the whole cigarette. More recently, yields per milligram of nicotine have been considered, which also is a mathematically derived result, but in this case, the results of the yield per milligram of tar is extrapolated to the nicotine content determined in a separate assay (95). There are rationales, merits, and limitation for all these methods. The results on a per milligram of tar basis reflects the potency of the nonvolatile fraction of the smoke, and can be considered as raw data and the best direct comparison of tobacco products. However, how a smoker will puff their cigarettes, including ventilation hole blocking, can substantially change the character and proportion of the chemical constituent yields, and so the potency of the smoke only reflects that particular puff profile used to generate the smoke. The yield per cigarette seemingly makes sense for comparing one cigarette to another, but its limitation is that a specific number of puffs is assumed or related to changes in the smoking parameters that is not representative of the number of puffs that a smoker would actually inhale. Also, the same limitations as for the per milligram of tar basis apply to the per cigarette basis. Thus, the per cigarette calculation represents the limitations of the per milligram basis compounded by variables that affect puff number. Reporting results on a per nicotine basis is based upon the assumption that people smoke their cigarette to titrate blood

[8] http://www.coresta.org/Reports/IVT_TF_Report_Smoke_Air_Liquid_Interface.pdf

Table 3. History of manufactured Kentucky reference cigarettes

Name	Year produced	Tar mg/ cigarette	Nicotine mg/ cigarette	Description
1R1	1969	30.1	1.98	Original reference cigarette
2R1	1974	32.9	2.19	Followed the 1R1
2R1F	1974	23.4	1.74	Filtered version of the 2R1
1R3	1974	20.3	1.23	Equivalent of the standard experimental blend used by the National Cancer Institute
1R3F	1974	15	1.16	Filtered version of the 1R3
1R5F	1989	1.67	0.16	First ultralow yield cigarette
1A1	1969	N/A	N/A	Low nicotine yields
2A1	1974	31.8	0.42	Followed the 1A1
3A1	1974	26.8	0.24	Followed the 2A1
1A2	1969	29.5	1.75	High intermediate nicotine delivery, compared with 1A1
1A3	1969	25.8	1.14	High low intermediate nicotine delivery, compared with 1A1
1A4	1969	29.8	2.2	High nicotine delivery, compared with 1A1
1R4F	1983	9.2	0.8	Lower yield nicotine research cigarette
2R4F	2001	9.2	0.8	Follows the 1R4F
3R4F	2008	9.4	0.72	Follows the 2R4F

NOTE: http://www.ca.uky.edu/refcig/.
Abbreviation: N/A, not available.

nicotine levels and theoretically represents a calculation to correct for a smoker's nicotine intake. However, this is a similarly contrived calculation as the per cigarette analysis, because the nicotine yields vary according to the machine smoking parameters, just like tar yields vary, incorporating the limitations of the per milligram of tar basis compounded by the assumptions around the number of puffs.

Reference (Control Cigarettes). The availability of reference cigarettes to use as controls in experiments allows for quality control within laboratories and for interpreting data from different laboratories. Although some studies have sought to use commercial cigarette as both test samples and controls, these are not common, and the composition of commercial cigarettes changes frequently with the changing marketplace, making comparisons of results difficult. To address this issue, a series of research cigarettes have been made available through the University of Kentucky Reference Cigarette Program, which are only changed every several years.[9] Table 3 provides a history of the available Reference Cigarettes as compiled from various sources (30, 96).[10] Currently, the following cigarettes are available: 1R5F, cigarette designed with a tar and nicotine FTC delivery of 1.67 and 0.16 mg, respectively, and the 3R4F, which yields 9.4 mg tar and 0.726 mg nicotine.[9]

Preparation of ST Extract (STE) for Toxicology Testing. There are many different approaches for analyzing ST for *in vitro* studies that use some type of extraction method to make STE. In general, the procedure begins with grinding the tobacco to a fine powder, sometimes following a freeze drying step, and then the powder is suspended in extraction medium and centrifuged. The STE is then filtered to remove the undissolved materials. ST has been extracted with water (97-99), cell culture media (100-111) or buffers such as HANKS-balanced salt solutions (112, 113), PBS (114-116), or saline (117). Artificial saliva also is sometimes used (118). Such STEs can then be lyophilized and redissolved to concentrate them (119, 120). Other methods for ST extraction include direct extraction in DMSO (118) or solvents such as methylene chloride. (118, 121-123), followed by evaporation under reduced pressure and redissolution in ethanol (122) or DMSO (121). Modifications to methylene chloride extraction protocols have also been made using additional extraction in methanol and acetone before final dissolution in DMSO, because methylene chloride can itself be mutagenic (118, 123). Successive filtration can be used (115-117), and the filtrate can be lyophilized to increase the concentration (114-116). Some modifications have been made to ST extraction for Ames testing (119, 122, 124).

Most STE toxicology assay results are reported on a dry weight basis, as specified by the FTC.[11] In these methods, the dry weight is determined by drying the ST with heat, although the original nondried ST actually is used to generate the extracts for assay. Calculating the dry weight, however, only standardizes results based on water and volatiles content, and does not account for differences in humectant and solids levels, which can vary widely among ST products. Currently, there are no published methods or standards for assaying ST applying a correction for moisture and humectants. An alternative method for reporting ST toxicology results would be on a per milligram of nicotine of wet ST, under the assumption that ST users will titrate their ST use based on their nicotine needs. Optimally, extracts would be prepared and analyzed using approaches that mimic human use. However, ST topography studies have not been conducted on which such methods could be based.

ST reference control products have been developed by the University of Kentucky, Tobacco and Health Research Institute in the late 1980s. These reference products were custom made to mimic the chemical composition of commercial moist snuff (1S3), dry snuff (1S2), and loose-leaf snuff (1S1). The flavorings and additives, including those included by the manufacturer to influence levels of unprotonated nicotine, were not included. Today, these reference products are old and have not been replaced, and are only available from the North Carolina State University

[9] http://www.ca.uky.edu/refcig/
[10] http://www.ca.uky.edu/refcig/; http://tobaccodocuments.org/ctr/60039113-9121.html; http://tobaccodocuments.org/ctr/60039113-9121.html
[11] http://edocket.access.gpo.gov/2009/pdf/E8-31465.pdf

Table 4. Kentucky reference ST products (126)

		Tobacco by weight	Nicotine	Year
1S1	Loose-leaf	40%	0.95	1987
2S1	chewing tobacco	40%	0.84	Current version of 1S1
1S2	Dry snuff	75%	1.60	1987
1S3	Moist snuff	37%	2.51	1987
2S3		37%	1.34	Current version of 1S3

Department of Crop Sciences (P.O. Box 7620 4324 Williams Hall Raleigh, NC 27695-7620). Some specific characteristics of each reference snuff type are indicated in Table 4 (121, 125, 126).

***In vitro* Toxicology Testing.** A wide variety of *in vitro* assays have been used over the years to assess the toxicity of cigarette smoke and ST extracts. These assays have amply shown that these materials can produce dramatic effects on cells of various types, resulting in altered cellular viability and proliferation, inducing DNA damage, altering cellular behavior, and changing the pattern of gene expression and protein production. What is less clear, however, is how the results from these assays should be interpreted and what information they provide in the context of human toxicity. In the following sections, we examine various types of assays that have been used in tobacco product testing. We describe what these assays are intended to measure, how they work, and explore considerations that impact assay design and the interpretation of the data. As this publication will show, most *in vitro* testing has been limited to a small number of cytotoxicity and genotoxicity assays. There are only isolated studies that have compared one type of tobacco product to another, including for PREPs, and virtually all studies generate TPM or CSC using the FTC machine smoking method. The choice of the cells to be used is a critical determinant for the design and interpretation of *in vitro* toxicology assays. A separate consideration is that almost all *in vitro* assays can be confounded by cytotoxicity, and therefore these tests are usually conducted alongside other assays, but there is little guidance on what is an acceptable level of cytotoxicity.

Cytotoxicity. An evaluation of general toxicity through cell death is probably the most common assessment made for tobacco products. Typically, assays of this type are done for one of two reasons: (*a*) to simply characterize the toxicity of the materials in one or more cell culture systems or (*b*) to determine the maximum doses of the test materials that can be used in other assays without causing too much cell death. In this review, assays that are designed to measure the induction of programmed cell death (e.g., apoptosis) will be considered separately; although there is considerable overlap between apoptosis assays and simple cytotoxicity assays, and the distinction between these processes is much less clear than is frequently portrayed.

One rationale given for the measurement of the cytotoxic activity of cigarette smoke and ST extracts in the context of cancer risk assessment is the notion that toxicity is somehow related to the carcinogenic potential of the material. This is based on the assumption that there are some shared mechanisms for cytotoxicity that relate to mutagenicity and carcinogenicity. Cytotoxicity assays, however, measure insults that are sufficient to kill or severely damage the cells, and dead cells cannot transform and have malignant potential. Thus, they measure much more than effects that contribute to carcinogenesis. For cytotoxicity assays to be useful tools for comparing the potency of different tobacco products, one therefore has to make the assumption that the level of sublethal (procarcinogenic) damage produced by lower test doses is somehow proportional to the dose required to produce overt cytotoxicity. It is far from clear that this assumption is valid and is an important issue that needs to be resolved, particularly if assays of this type are to be used to evaluate the potential for cancer risk reduction through the use of PREPs.

There are many approaches that can be used to measure cytotoxicity, accounting for a variety of cellular and biological end points. These end points can include a loss of plasma membrane (PM) integrity, damage to intracellular membranes, loss of intracellular biochemical functions, degradation of intracellular biochemical gradients, and/or a complex combinations of these and other effects. Given the plethora of cytotoxicity outcomes that can be assessed by various researchers, comparisons among studies can be challenging. Thus, it is important to have some understanding of the mechanisms upon which a given assay is based, as well as how any given assay can be affected by the experimental conditions and some unforeseen effect by the test materials unrelated to cytotoxicity. For example, if cytotoxicity is assayed by measuring active enzyme release into culture medium, then it is important to know that the tobacco toxicants do not inhibit the activity of that enzyme. Similarly, assays that depend on colorimetric or fluorescence-based end points can be compromised if the tobacco extract absorbs or fluoresces at the same wavelengths as the assay's reporter components. Such confounding effects can be controlled if anticipated, e.g., by the use of several assays that depend upon different mechanisms and modes of action. A separate consideration is that cytotoxicity is almost always a function of both the dose of the material being tested and the time for which the cells are exposed to the test material, where longer exposures can affect the dose-response relationship. Length of time for exposure is rarely addressed in toxicology studies, rather the length of time is usually arbitrarily determined. This effect is further complicated by the possibility that metabolic processes can be affected directly or by changes in proliferation rates or cell cycle, dependent on dose, so that the metabolism of tobacco toxins may be increased or decreased, again affecting the cytotoxicity outcomes.

Table 5 provides a list of studies that have assessed cigarette smoke cytotoxicity, either as TPM, CSC, WS, or GVP. (Because TPM and CSC have been used inconsistently in the literature, we also include the actual method, e.g., cold trap or filter pad). There are very different levels of sensitivity for various assays and cell systems, and doses range widely depending on the test system. As shown in Table 5, most studies use TPM or CSC generated by the FTC smoking machine method. Other puffing regimens and comparisons of cigarettes with different filter designs provide consistent results for the direction of change, e.g., increased or decreased cytotoxicity for changing parameters such as puff volume, but the increase or decrease depends on how the data are reported

(e.g., per milligram of tar or per cigarette basis). For example, TPM and GVP from Marlboro ultralights had more cytotoxicity than Marlboro Lights on a per milligram of TPM basis, a trend that paralleled a statistically significant increase of chemical constituents, but was statistically reduced on a per cigarette basis (127). Human studies, however, do not show differences in exposure for these types of cigarettes, indicating the complexity of extrapolating *in vitro* cytotoxicity assays to human exposure (33, 128). Several laboratories also have studied the cytotoxicity of GVP and WS, which are reported to be more cytotoxic than TPM or CSC when using short-term exposures, although other studies do not show this (72, 89, 129, 130). There are some publications from tobacco companies showing that various tobacco ingredients and cigarette paper design, such as licorice extracts, glycerin, vanillin, and potassium sorbate have no effect on cytotoxicity for TPM, CSC, GVP, or WS (68, 131-134). Internal company documents show that other ingredients and technologies can affect cytotoxicity, but generally, these are not marketed.

Cytoxicity studies for STE are shown in Table 6. STE is less cytotoxic than TPM and/CSC, but the cytotoxicity varies among ST products.

Plasma Membrane Permeability Cytotoxicity Assays. Probably the simplest types of cytotoxicity assays are those that measure the permeability of the cellular PM, a sign of dead cells. For the assessment of tobacco products, available studies are shown in Table 5. However, the sensitivity and confounding variables for most available PM assays have not been tested. The classic way to assess PM is by the trypan blue exclusion assay, where an intact PM does not allow the dye to enter the cell, but the dye diffuses in when damaged. Under the microscope, viable cells remain clear, whereas those with compromised PMs turn blue. This assay is simple and the reagents are inexpensive, but it is relatively time consuming and not readily adapted for use in high throughput assays. The trypan exclusion assay has been used to asses both WS and TPM, and dose-dependent results were found (55, 135). Similar assays have high throughput assays that also assess dye exclusion using fluorescent DNA stains, such as ethidium bromide or propidium iodide (PI), and measured by flow cytometry or in fluorescence-based 96-well plate assays (76, 103, 113). Another approach for assessing PM integrity is the lactate dehydrogenase (LDH) release assay that measures the amount of LDH in the media that has escaped through damaged membranes. In this case, activity is measured by the reduction of NAD and stoichiometric conversion of a tetrazolium dye measured at a wavelength of 490 nm (44). The LDH release assay is among the most sensitive cytotoxic methods for short exposure times, for example when used in Chinese Hamster ovary cells (CHO; ref. 44). An alternative to assessing LDH activity in the medium is to remove the media and measure the remaining LDH activity in the cells, but this must be done with an assessment of cell number, because lower numbers will lead to false negative results (136). Another class of PM permeability assay depends on the activity of nonspecific intracellular esterases within the cell. Nonfluorescent esterase substrates such as Calcein alveolar macrophage (AM) can passively diffuse into viable cells and esterases convert them into charged florescent products that are trapped in the cell, but a damaged PM allows these products to diffuse out of the cell and so cell viability can be assessed by cell fluorescence (137). These PM assays can be confounded by substances that inhibit enzyme activity and also those that have absorbance at 490 nm, both of which can happen with TPM and CSC.

Assays for pH Gradients as Cytotoxicity Assays—Neutral Red Uptake. Probably the most widely used assay for the assessment of cytotoxicity in the context of tobacco product testing has been the neutral red uptake (NRU) assay, as indicated in Table 5. Neutral red is an acidotropic stain that is taken up by lysosomes. The maintenance of pH gradients within the cell requires intact ATP-dependent proton pumps, and so cytotoxic insults that damage lysosomal membranes or cause interruption of normal energy-requiring cellular processes will decrease the uptake and binding of the dye (19, 44). In brief, this assay is done by adding the neutral red solution to culture medium and incubating the cells. The cells are then fixed and the dye is solubilized; the amount of uptake is measured at a wavelength of 540 nm. This assay has been recommended for inclusion in a battery of *in vitro* assays to evaluate the biological activity of CSC (19). Protocols have been written and reviewed (138).[12] Many laboratories conduct this assay with CHO or Mouse embryo BALB/c cells (43, 50, 59-61, 72, 86, 129, 139-141). In doing this assay, it needs to be noted that prolonged exposure of the cells to the fixative can result in leaching of the dye into the solution. Another limitation is that the neutral red may precipitate in solution and interfere with the assay, and again, test materials that have absorbance maxima near 540 nm may complicate assay design. Other acidotrophic stains such as acridine orange and a range of newer fluorescent probes can be used in a similar context and may allow the use of wavelengths that can circumvent this problem, but have not been widely used for tobacco assessments.

Redox Potential–Based Cytotoxicity Assays. Another commonly used class of cytotoxicity assays is based on the production of formazan dyes from tetrazolium salts that are reduced by cellular dehydrogenase enzymes (these same assays can be used to assess cellular proliferation, see below). Specifically, the MTT, 2-(4-iodophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H tetrazolium (WST-1), and 2-(2-methoxy-4-nitrophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H-tetrazolium (WST-8) assays incubate cells with MTT, WST-1, or WST-8, respectively. Then, spectral absorbance at 450 nm is determined for the release of formazan dye. These assays are easy to perform and are adaptable for high throughput analyses. An advantage is that the assays do not need centrifugation or fixation of cells, which can decrease random error and enhance reproducibility and reliability. The technical limitations, however, relate to metabolic interference, such as the generation of formazan by reducing agents and chemicals that affect mitochondrial dehydrogenase activity (142). The WST-1 and WST-8 assays are considered to be advances on the MTT assay in general, because they have fewer steps, are more stable, and have a wider linear range of color; the WST-8 assay is more sensitive than the

[12] http://www.hc-sc.gc.ca/hc-ps/tobac-tabac/legislation/reg/indust/method/tox-eng.php

Table 5. Cytotoxicity assays for cigarette smoke (CSC/TPM), WS, and gas vapor phase smoke

Assay	End point measured	Cell studied	Smoke conditions
Acid phosphatase activity	Cell membrane acid phosphatase activity	CHO WBL strain	FTC
Alamar blue assay	Mitochondrial dehydrogenase activity	Human bronchial epithelial cell line HBE1	35-50 mL puffs within 2 min
ATP bioluminescence assay	Intracellular ATP content	3PC keratinocytes and J774A.1 macrophages	FTC
^{3}H-adenine release	Membrane integrity	Rat Alveolar type II cells	N/A
Cell viability assay	Count of live and dead cells by automated counter	Human fetal bronchial epithelial cells	FTC
CytoTox 96 cytotoxicity assay	LDH release into medium	Human aortic endothelial cells	N/A
Dye-exclusion assay ViaCount	Flow cytometry	Human airway epithelial cells, primary and transformed	MDPH
LDH release	Amount of LDH released as a result of membrane damage	Guinea pig and human AMs	FTC
		Pig pulmonary artery endothelial cells	N/A
		Human foreskin, bronchial epithelial, coronary endothelial, coronary smooth muscle, and rat liver epithelial cells	FTC
		Beas-2B cells	N/A
		CHO WBL strain	FTC
		Mouse lung epithelial cell line LA-4 cells	ISO
		Lung epithelial cells	ISO
		Murine 3T3 fibroblasts	ISO and an intense smoking condition
		Normal human bronchial epithelial cells	FTC
		Human gingival fibroblasts	N/A
MTT	Reduction of tetrazolium salts to formazan dyes by cellular enzymes	Human normal breast epithelial cell line MCF10A	FTC
		Human epidermal keratinocytes and oral carcinoma cells	FTC
		Human lung epithelial cell line A549	N/A
		Human pulmonary artery endothelial cells	N/A
		Human lung epithelial cells A549	N/A
Neutral red	Measure the amount of dye incorporated into lysosomes	CHO-WBL	FTC
		CHO WBL strain	FTC
		CHO WBL strain	FTC
		CHO WBL strain	FTC
		Mouse embryo BALB/c 3T3 cells	ISO
		CHO WBL strain	FTC
		Mouse embryo BALB/c 3T3 cells	FTC
		Human lung epithelial cell line A549	ISO

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Table 5. Cytotoxicity assays for cigarette smoke (CSC/TPM), WS, and gas vapor phase smoke (Cont'd)

Cigarette type	Test substance*	Cytotoxicity reported in study	Reference
1R4F	TPM (Cambridge method)	Cytotoxic effect observed	Putnam, KP 2002 (44)
N/A	GVP (Exposure chamber)	Little effect at 1 h, but increased response with greater dose at 24 h	Spencer, JP 1995 (93)
1R4F,ECLIPSE	CSC (Cold trap)	Concentration-dependent response observed, eclipse CSC exhibited reduced cytotoxicity	Curtin, GM 2004 (272)
1R1 cigarette	WS (Solvent trap)	Cigarette smoke treated medium showed no cytotoxicity	Wirtz, HR 1996 (74)
Research cigarette type A and B	WS (exposure chamber), smoke diluted with synthetic air	Different effects by air dilution and cigarette type	Ritter, D 2003 (85)
1R4F	TPM (Cambridge method)	Minimal cytotoxicity	Nordskog, BK 2003 (136)
Major U.S. brand of filtered, "full-flavor" cigarette	WS (Jet impaction) and GVP (Solvent trap)	Concentration-dependent response differed by cell types	Hays, LE 2008 (162)
Commercial cigarette, brown tobacco, without filter	GVP (exposure chamber)	The LDH release from both cells exposed to smoke and to purified air were not significantly different	Dubar, V 1993 (91)
Commercial Marlboro cigarette	WS (Solvent trap)	No cytotoxic effect was observed	Su, Y 1998 (71)
1R4F, 1R5F, and a cigarette that primarily heats tobacco (TOB-HT)	TPM (Cambridge method)	1R4F and 1R5F increased cytoxicity. TOB-HT did not show effect in any of the cell types tested.	McKarns, SC 2000 (50)
Kentucky reference 1R3	GVP (exposure chamber)	Gas phase smoke-induced cytotoxicity	Arora, A 2001 (90)
1R4F	TPM (Cambridge method)	LDH release was most sensitive assay after 1 h of exposure	Putnam, KP 2002 (44)
Cigarettes with different filters	GVP (exposure chamber)	Cytotoxicity differed by filter type	Pouli, AE 2003 (94)
A major international brand	GVP (exposure chamber)	Dose- and time-dependent response observed	Piperi, C 2003 (92)
Dark, American, and Virginian with standard and modified cellulose acetate filters	CS (Solvent trap) and GPCS (Solvent trap)	Time-dependent response observed, CS and GPCS induced different cytotoxicities	Culcasi, M 2006 (88)
2R4F,Quest low nicotine and nicotine free	TPM (Cambridge method)	Dose response observed but no significant difference among cigarettes	Chen, J 2008 (146)
N/A	CSC	Dose-dependent increase in cytotoxicity observed	Zhang,W 2009 (151)
1R4F	TPM (Cambridge method)	Concentration-dependent response observed.	Narayan, S 2004 (21)
1R3F	CSC (Cambridge method)	Concentration- and time-dependent response observed	Nagaraj, NS 2006 (48)
An Indian cigarette with filter	CSC (Solvent trap)	Concentration response observed	Kaushik, G 2008 (76)
Long Life cigarettes	CSE (Cambridge method)	Dose-dependent increase observed	Hsu, CL 2009 (159)
Commercial filtered cigarette	AECS (Solvent trap)	Dose- and time-dependent increase observed	Das, A 2009 (152)
Commercial and novel carbon filter cigarettes	TPM (Cambridge method) and WS (Exposure chamber)	Dose-response reported for CSC but no difference between cigarettes. WS of novel carbon cigarettes was less cytotoxic.	Bombick, DW 1997 (59)
1R4F, 1R5F, and a cigarette that primarily heats tobacco (TOB-HT)	WS (Exposure chamber)	WS from the 1R4F cigarette was significantly more cytotoxic on a per cigarette basis than the smokes from the 1R5F and TOB-HT cigarettes	Bombick, DW 1998 (86)
Kentucky 1R4F and 1R5F, a cigarette primarily heats tobacco (TOB-HT)	TPM (Cambridge method)	Dose-dependent response observed for reference cigarettes, but not for TOB-HT	Bombick, BR 1997 (60)
1R4F	TPM (Cambridge method)	Concentration-dependent response observed	Putnam, KP 1999 (43)
1R4F, cigarettes with different flavoring and tobacco	TPM (Cambridge method) and GVP (Solvent trap)	No differences in GVP cytotoxicity, but there were some differences for TPM among the cigarettes	Roemer, E 2002 (72)
1R4F	TPM (Cambridge method)	Cytotoxic effect observed. Most sensitive following 24 h of exposure.	Putnam, KP 2002 (44)
1R4F,an EHC(EHC)	TPM (Cambridge method) and GVP (Solvent trap)	TPM and GVP from EHC were less cytotoxic than 1R4F on a per cigarette basis	Tewes, FJ 2003 (89)
2R4F, 2R4F with modified acetate or charcoal filter	WS and GVP (Exposure chamber)	GVP less cytotoxic than WS. Charcoal filter decreased the cytotoxicity of GVP	Fukano, Y 2004 (83)

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Table 5. Cytotoxicity assays for cigarette smoke (CSC/TPM), WS, and gas vapor phase smoke (Cont'd)

Assay	End point measured	Cell studied	Smoke conditions
Neutral red	Measure the amount of dye incorporated into lysosomes	Mouse embryo BALB/c 3T3 cells	FTC/ISO, MDPH
		CHO-WBL cells	FTC, HC, MDPH
		BALB/c 3T3	ISO
		BALB/c 3T3	ISO
		Normal human bronchial epithelial cells	FTC
		Mouse embryo BALB/c 3T3 cells	FTC
		CHO K1	ISO
		CHO	FTC
		CHO	ISO, HC
		CHO	FTC
		BALB/c 3T3	ISO
		BALB/c 3T3	ISO
		Human lung carcinoma cell line NCI-H292 and A549	ISO
		BALB/c 3T3 clone A31	ISO
		Mouse embryo BALB/c 3T3 cells	FTC/ISO, MDPH, human puff profile (HPP)
		Mouse embryo BALB/c 3T3 cells	ISO, HPPs
		Human bronchial epithelial cell line BEAS-2B	FTC
		CHO	FTC
		Mouse embryo BALB/c 3T3 cells	FTC with modifications for tar production

(*Continued on the following page*)

Table 5. Cytotoxicity assays for cigarette smoke (CSC/TPM), WS, and gas vapor phase smoke (Cont'd)

Cigarette type	Test substance*	Cytotoxicity reported in study	Reference
Eight commercial cigarettes, three reference cigarettes, EHC	TPM (Cambridge method) and GVP (Solvent trap)	MDPH showed higher cytotoxicity on a per cigarette basis. The opposite was found on a per milligram basis, although the results may not be stat. significant. EHC less cytotoxic than commercial cigarettes. GVP was more or less cytotoxic than TPM depending on the reporting method and product.	Roemer, E 2004 (139)
Ultralights and Eclipse	TPM (Cambridge method)	Ultralights were cytotoxic, whereas Eclipse was not	Foy, JW 2004 (61)
1R4F, test cigarette containing typical commercial tobacco blend no additives with cellulose acetate filters	TPM (Cambridge method) and GVP (Solvent trap)	No difference seen between licorice treated and control cigarettes	Carmines, EL 2005 (132)
1R4F, test cigarette containing typical commercial tobacco blend no additives with cellulose acetate filters	TPM (Cambridge method) and GVP (Solvent trap)	No difference seen between glycerin treated and control cigarettes	Carmines, EL 2005 (131)
1R4F, Eclipse	CSC (Cambridge method)	Concentration dependent decrease observed with 1R4F, no decrease observed with Eclipse	Fields, WR 2005 (155)
1R4F and 2R4F	TPM (Cambridge method) and GVP (Solvent trap)	There were no statistically significant differences between the cigarettes	Counts, ME 2006 (130)
Dark, American, and Virginian with standard and modified cellulose acetate filters	CS (Solvent trap) and GPCS (Solvent trap)	Modified cellulose acetate filters which included charcoal showed significantly lower EC_{50} values than modified cellulose acetate filters without charcoal in GPCS	Culcasi, M 2006 (88)
Cigarettes with different levels of fructose corn syrup and reference cigarettes	CSC (Cambridge method), WS (Exposure chamber)	No significant differences for CSC and WS of cigarettes tested	Stavanja, MS 2006 (140)
Kentucky reference 1R4F and 2R5F,Canadian Monitor	TPM (Cambridge method) and GVP (Solvent trap)	TPM for HC less cytotoxic on a per milligram basis. GVP more cytotoxic for HC	Rickert, WS 2007 (141)
Standard American tobacco blend, three test cigarettes with banded cigarette paper technologies and control cigarette	CSC (Cambridge method) and WS (Exposure chamber)	No significant difference in cytotoxicity observed between control and test cigarettes in both CSC and WS	Theophilus, EH 2007 (68)
Typical American blend with and without vanillin	TPM (Glass filter) and GVP (Solvent trap)	No statistically significant differences seen between test and control cigarette	Lemus, R 2007 (134)
Typical American blend with and without potassium sorbate, 2R4F	TPM (Glass filter) and GVP (Solvent trap)	No statistically significant differences seen between test and control cigarette	Gaworski, CL 2008 (133)
2R4F	TPM (Cambridge method)	Similar cytotoxicity was observed for both cell lines at highest test dose	Newland, N 2008 (273)
1R4F, three commercial cigarettes	TPM (Cambridge method) and GVP (Solvent trap)	TPM from two and GVP from one of the commercial cigarettes were observed to be significantly different from the 1R4F when reported on a per cigarette basis	Patskan, GJ 2008 (127)
Two EHC prototypes, eight commercial cigarettes, and K1R4F	TPM (Cambridge filters)	Cytotoxicity from EHC was 50-75% lower per milligram of TPM, less than 50% per milligram of nicotine, and 95% lower on a per cigarette basis compared with commercial cigarettes	Roemer, E 2008 (224)
Two Marlboro brands, reference cigarette, EHC	TPM (Cambridge method) and GVP (Solvent trap)	GVPs generated under ISO conditions showed less cytotoxicity than those by HPP on a per cigarette basis. EHC GVP and TPM less cytotoxic.	Werley, MS 2008 (129)
2R4F	CSC (Cambridge method)	Time- and concentration-dependent decrease observed	Carter, CA 2009 (160)
Reference cigarette, commercial tobacco blend, with and without cast sheets	TPM (Cambridge Method) and WS (Exposure chamber)	Concentration-dependent increase observed with both CSC and WS.	Potts, RJ 2009 (84)
1R5F, 2R4F, 2R1F	TPM (Glass fiber filter) CSC (impaction trap)	Concentration-dependent decreases in viability observed, cigarettes smoked to the same TPM yield showed different cytoxicities	Roemer, E 2009 (274)

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Table 5. Cytotoxicity assays for cigarette smoke (CSC/TPM), WS, and gas vapor phase smoke (Cont'd)

Assay	End point measured	Cell studied	Smoke conditions
Nigrosin staining	Dye enters damaged cells	Polymorphonuclear blood cells	FTC
Resazurin binding	Metabolic activity of living cells	CHO WBL strain	FTC
Sulforhodamine B binding	Amount of dye removed estimates number of viable cells	CHO WBL strain	FTC
trypan blue dye exclusion	Dye enters damaged cells, healthy cells stay white.	Mouse L-929 cells, a fibroblast-like cell line	Puff number and puff volume varied
		Human lung epithelial cell line A549	FTC
WST-1	Reduction of tetrazolium salts to formazan dyes by cellular enzymes	Lung epithelial cells	ISO
		Mouse lung epithelial cell line LA-4 cells	ISO
		Human fetal bronchial epithelial cells	FTC
		Human lung epithelial cell line A549	ISO
		Human gingival fibroblasts	N/A
WST-8	Reduction of tetrazolium salts to formazan dyes by cellular enzymes	Normal human bronchial epithelial cells	FTC
XTT assay	Activity of mitochondrial dehydrogenases	CHO WBL strain	FTC

*TPM and CSC as reported in publication, with the actual method indicated in parenthesis.

WST-1 assay (143). Although the MTT assay is less expensive and the MTT may still be useful for tobacco studies because the purple formazan produced by this reagent allows for measurements at wavelengths less likely to be confounded by tobacco extracts colors.

Novel cytotoxicity assays are being developed that may be adaptable for the assessment of tobacco products. For example, Lindl and coauthors (143) evaluated the *in vitro* cytotoxicity of 50 chemicals using an electrical current exclusion method that is based on the different electrical properties of dead and viable cells. Cells are suspended in an isotonic and iso-osmotic electrolyte solution, separated, and exposed to a low-voltage field. The cells' electrical signal is proportional to the cell volume and membrane permeability; live cells act as insulators and dead cells are permeable to the electric current. Therefore, live and dead cells can be differentiated by the different electrical signals generated per cell volume. Lindl (143) reported that the electrical exclusion method was more sensitive than the NRU and WST-8 assays, and found a high correlation between the IC_{50} values obtained with these assays.

STE Cytotoxicity Studies. Cytotoxicity for STE has been studied in a number of cell lines as shown in Table 6, and although there is some conflicting data depending on the *in vitro* model, it is possible to elicit a cytotoxic response. A few studies have used human cells, such as lymphoblast cells lines, colon carcinoma lines, and skin fibroblasts (98, 117). One study has used human oral keratinocytes, which at least comes from the target organ (144). Most studies investigate the cytotoxicity of the Kentucky reference moist snuff or chewing tobacco, whereas there have been only limited applications for commercially available products (98, 105, 107, 118). At this point, there is very little information about the cytotoxicity of products that are available on the market and currently being used.

PREP Cytoxicity Studies. Cytotoxicity assays have been applied to different combustible PREPs, and these studies also are listed in Table 5; virtually all of the studies were done at tobacco company laboratories. For example, using the FTC smoking machine methods, Eclipse cigarettes were reported to yield less or no cytotoxicity in different cell types compared with reference or other commercially available cigarettes (50, 60, 61, 86, 139, 145). Although the cytotoxicity of TPM, CSC, and GVP generated from an electrically heated cigarette (EHC) prototype under two different smoking machine conditions were reported to be lower compared with conventional and reference cigarettes on a per cigarette basis, but not on per milligram TPM/CSC basis (139). In another study, WS from the Eclipse cigarette under the FTC conditions showed more cytotoxic activity in CHO cells than the 1R5F cigarette (an ultralight reference cigarette), although it showed less cytotoxicity than a1R4F cigarette (86). Furthermore, GVP from an EHC reportedly showed less cytotoxicity than that of 1R4Fon a per cigarette basis (89). Assays also were done to evaluate the effect of new filter designs on the cytotoxicity of cigarette smoke. It was shown that the WS of a new carbon filter cigarette was less toxic than cigarettes with other filters (59). In another study, GVP from a cigarette with a cellulose acetate and charcoal filter reportedly showed less cytotoxicity than that from cigarette with a cellulose acetate filter (88). A filter with three different types of activated carbon also was reported to be capable of reducing cytotoxicity (94).

Table 5. Cytotoxicity assays for cigarette smoke (CSC/TPM), WS, and gas vapor phase smoke (Cont'd)

Cigarette type	Test substance*	Cytotoxicity reported in study	Reference
British cigarette high tar, middle tar, and low tar	CSC	Control cells from heavy smokers without respiratory disability were more resistant to cytotoxicity than those from heavy smokers with severe disease	Hopkin, JM 1981 (275)
1R4F	TPM (Cambridge method)	Cytotoxic effect observed	Putnam, KP 2002 (44)
1R4F	TPM (Cambridge method)	Cytotoxic effect observed	Putnam, KP 2002 (44)
Different filter types	WS (Exposure chamber)	Cytotoxicity decreased with increasing smoke age and increased with puff numbers and increasing amounts of charcoal in filter	Sonnenfeld, G 1985 (135)
2R4F	TPM (Cambridge method)	Dose-dependent response observed	Fukano, Y 2006 (55)
A major international brand	GVP (Exposure chamber)	Dose-dependent response observed	Piperi, C 2003 (92)
Cigarettes with different filters	GVP (Exposure Chamber)	Cytotoxicity differed by filter type, dose- and time-dependent response observed	Pouli, AE 2003 (94)
Research cigarette type A and B	WS (Exposure chamber), smoke diluted with synthetic air	Different effects by air dilution and cigarette type	Ritter, D 2003 (85)
2R4F, 2R4F with modified acetate or charcoal filter	WS and GVP	GVP less cytotoxic than WS, charcoal filter decreased the cytotoxicity of vapor phase.	Fukano, Y 2004 (83)
N/A	CSC	Cytotoxicity increased in a dose-dependent manner	Zhang, W 2009 (151)
2R4F,Quest low nicotine and nicotine free	TPM (Cambridge method)	Dose-response observed, differences in cytotoxicity existed at some concentrations	Chen, J 2008 (146)
1R4F	TPM (Cambridge method)	Cytotoxic effect observed after 12-24 h of exposure	Putnam, KP 2002 (44)

In other studies, TPM/CSC from a nicotine-free product were not less cytotoxic than reference or low-nicotine cigarettes (146). Thus, under FTC conditions, it is possible to differentiate the cytotoxicity among different PREPs and conventional products.

In comparison to PREP cigarette studies, there are even fewer studies reporting the *in vitro* cytotoxicity of smokeless PREPs (107, 118, 147, 148). The low cytotoxic capacity for ST using the NRU, and the low sensitivity of cytotoxicity assays, makes the study of STE cytotoxicity of limited utility for comparison of ST products (118). Results can differ when using wet or dry weights, and might be affected as well by humectants. There are no published studies for the cytotoxicity of STE prepared from low TSNA tobacco (e.g., snus) manufactured in the United States.

Cell Proliferation Assays. Cell proliferation reflects an increase in cell numbers as the result of cell growth and division, which is normally tightly regulated. Uncontrolled proliferation is one hallmark of malignant progression, although proliferation itself may not be a marker of malignant progression. Rapid proliferation exacerbates cell damage as it reduces time for metabolism and DNA repair between cell divisions, and so increases the risk for a cell to accumulate mutations. Cell proliferation is affected by growth factors, growth factor receptors, and other signaling and transcription factors, and so genotoxic damage to genes controlling these pathways can alter cell proliferation. Agents that can induce cell proliferation are sometimes called mitogenic in the context of triggering mitosis. Because proliferation rate is a function of cell division, a cell proliferation assay ideally measures the number of cells that are dividing in a culture at a given time. However, most commonly used proliferation assays do not measure cell division directly but rather estimate it, either by determining the number of cells that are synthesizing new DNA (a prerequisite for cell division) or by measuring the change in cell number in a culture over time. This distinction is important because it has implications for how the data generated are interpreted.

The studies in which proliferation assays have been used to asses cigarette products are shown in Table 7. TPM and CSC have been reported to increase cell proliferation at low concentrations, but it decreases at high concentrations due to cytotoxicity (149). Although there are numerous studies about individual tobacco products, and a few that assess different types of cigarette filters as indicated in Table 7 (83, 94), there are almost no studies of PREPs. In one study, Quest cigarettes have been tested and the higher nicotine cigarettes had increased proliferation (146). STEs, as shown in Table 8, also induce cell proliferation with a dose-dependent effect at lower concentrations in lymphocytes, epidermal keratinocytes, and fibroblasts, although proliferation also is reduced at higher concentrations (113, 116). STE of Swedish snus inhibited proliferation of both rat spleen and T cells in a dose-dependent manner (107). Although some of the assays might be easier to use than others, at the present time, there is little basis to recommend one for tobacco testing in particular, because there are no comparative studies indicating similar or different results.

DNA Synthesis–Based Proliferation Assays. These assays take advantage of the fact that a cell has to synthesize new DNA before replication. The level of new DNA synthesis is, therefore, a measure of the proliferative activity of a cell culture. Historically, the most commonly used DNA synthesis assay is the tritiated thymidine incorporation assay, where radioactive tritium-labeled

Table 6. Cytotoxicity assays used for ST extracts

Assay	End point measured	Cell studied	Extraction solution	ST product	Cytotoxicity reported in study	Reference
Cell survival assay	Counting of mutant cells resistant to purine analogue 6TG	Human lymphoblasts cell lines AHH-1 and TK-6	H_2O	Two American moist snuffs	Dose-dependent response. Both cell lines showed similar sensitivity	Shirname-More, L 1991 (98)
DAPI staining	Number of cells that incorporate fluorescent label	Human skin fibroblasts	DMEM	Commercial chewing tobacco	Dose- and time-dependent response observed, increase at low concentrations, cytotoxicity observed at higher concentrations	Coppe, JP 2008 (105)
LDH release	Amount of LDH released as a result of membrane damage	Macrophage J774A.1 cells	Phosphate buffer	Kentucky moist snuff	Concentration- and time-dependent increase in cytotoxicity observed	Bagchi, D 1995 (244)
		Golden Syrian hamster oral epidermoid carcinoma cell line	DMEM	1S3	No effect observed	Mangipudy, RS 1999 (103)
		CHO cells	H2O	Kentucky Reference chewing tobacco	No effect observed	Yildiz, D 1999 (99)
Neutral red	Measure the amount of dye incorporated into lysosomes	CHO cells	DMSO, DCM, artificial saliva	11 ST products	Concentration-dependent increase in cytotoxicity observed in DMSO extracted STE, Artificial saliva, and DCM showed no dose response	Rickert, WS 2009 (118)
MTS	Reduction of tetrazolium salts to formazan dyes by cellular enzymes	Golden Syrian hamster oral epidermoid carcinoma cell line	DMEM	1S3	Concentration-dependent response observed, differs by exposure time	Mangipudy, RS 1999 (103)
MTT	Reduction of tetrazolium salts to formazan dyes by cellular enzymes	Normal human oral keratinocytes	PBS	Kentucky moist snuff	Concentration- and time-dependent response	Bagchi, M 2001 (144)
		Oral leukoplakia cells (AMOL-III)	H_2O	Commercial khaini	Cytotoxicity was observed at higher concentrations	Rohatgi, N 2005 (97)
Trypan blue dye exclusion	Counting the number of cells that incorporate dye	Golden Syrian hamster oral epidermoid carcinoma cell line	DMEM	Reference Kentucky 1S3	No effect observed	Muns, G 1994 (106)
		Macrophage J774A.1 cells	Phosphate buffer	Kentucky moist snuff	Concentration- and time-dependent increase in cytotoxicity observed	Bagchi, D 1995 (244)
		HT-29 human colon adenocarcinoma epithelial cells	Saline	Reference Kentucky moist snuff	Concentration- and time-dependent response observed	Gregory, RL 1996 (117)
		Spleen cells, T cells and oral epithelial cells from rat	DMEM	Commercial Swedish moist snuff	Concentration- dependent response observed.	Hasseus, B 1997 (107)
		Normal human oral keratinocytes	PBS	Kentucky moist snuff	Concentration- and time-dependent response	Bagchi, M 2001 (144)

Abbreviation: DAPI, 4′,6-diamidino-2-phenylindole.

Table 7. Proliferation assays for cigarette smoke

Assay	End point measured	Cell studied	Smoke conditions	Cigarette type	Test Substance*	Proliferation results reported in study	Reference
Attachment assay	No. of cells that detach after treatment	Mouse embryonic fibroblasts, D3 mice embryonic stem cells	N/A	2R1, 1R4F, and four commercial cigarettes	MS (Solvent trap)	Dose-dependent response observed	Lin, S 2009 (161)
BrdUrd incorporation using Nova RED	Cells counted that dyed red	Human pulmonary muco-epidermoid carcinoma cell line NCI-H292, primary bronchial epithelial cells	N/A	Commercial cigarette and 1R3	CSC (Solvent trap)	Dose-dependent response observed at lower concentrations, proliferation decrease at higher concentrations	Luppi, F 2005 (73)
BrdUrd incorporation FACS analysis	Cells counted that incorporate BrdUrd	T cells extracted from peripheral blood mononuclear cells	N/A	1R4F	CSE (Solvent trap)	CSE treatment resulted in a significant reduction in proliferation	Glader, P 2006 (75)
^{3}H Thymidine uptake	Counting the number of cells that incorporate radioactive label	Alveolar type II epithelial cell line A549	N/A	Commercial medium tar cigarette	CSC (Solvent trap)	Dose-dependent response observed at higher concentrations	Lannan, S 1994 (150)
		Human lung epithelial cell line A549	N/A	An Indian cigarette with filter	CSC (Solvent trap)	Proliferation was increased at lower concentrations but was inhibited at higher concentrations.	Kaushik, G 2008 (76)
MTT	Reduction of tetrazolium salts to formazan dyes by cellular enzymes	Human normal breast epithelial cell line MCF10A	FTC	1R4F	TPM (Cambridge method)	Concentration-dependent response observed.	Narayan, S 2004 (21)
		Human epidermal keratinocytes and oral carcinoma cells	FTC	1R3F	CSC (Cambridge method)	Concentration- and time-dependent response observed	Nagaraj, NS 2006 (48)
		Human lung epithelial cell line A549	N/A	An Indian cigarette with filter	CSC (Solvent trap)	Concentration response observed	Kaushik, G 2008 (76)
		Human pulmonary artery endothelial cells	N/A	Long Life cigarettes	CSE (Cambridge method)	Dose-dependent decrease observed	Hsu, CL 2009 (159)
		Human lung epithelial cells A549	N/A	Commercial filtered cigarette	AECS (Solvent trap)	Dose- and time-dependent decrease observed	Das, A 2009 (152)
Survival/ Proliferation assay	Count the number of cells per colony using a computerized image analyzer	Human bronchial epithelial cells	N/A	N/A	CSC fractions	Dose-dependent inhibition of proliferation observed	Willey, JC 1987 (23)
Survival/ Proliferation assay	Counting colonies and stained cells	Wild-type and mutated CHO cell lines	N/A	1R4F	TPM (Cambridge method)	Concentration-dependent response observed differed by cell lines	Kato, T 2007 (52)
Survival/ Proliferation assay	Cells counted by Coulter counter	Normal human bronchial epithelial cells	FTC	2R4F,Quest low nicotine and nicotine free	TPM (Cambridge method)	Dose-dependent suppression of cell proliferation observed	Chen, J 2008 (146)
WST-1	Reduction of tetrazolium salts to formazan dyes by cellular enzymes	Lung epithelial cells	ISO	A major international brand	GVP (Exposure chamber)	Dose-dependent response observed	Piperi, C 2003 (92)
		Mouse lung epithelial cell line LA-4 cells	ISO	Cigarettes with different filters	GVP (Exposure chamber)	Proliferation differed by filter type	Pouli, AE 2003 (94)
		Human fetal bronchial epithelial cells	FTC	Research cigarette type A and B	WS (Exposure chamber), smoke diluted with synthetic air	Different effects by air dilution and cigarette type	Ritter, D 2003 (85)
		Human lung epithelial cell line A549	ISO	2R4F, 2R4F with modified acetate or charcoal filter	WS and GVP	Viability decreased as exposure increased, dose-dependent response observed	Fukano, Y 2004 (83)
		Human gingival fibroblasts	N/A	N/A	CSC	Higher concentrations decrease proliferation	Zhang, W 2009 (151)
WST-8	Reduction of tetrazolium salts to formazan dyes by cellular enzymes	Normal human bronchial epithelial cells	FTC	2R4F,Quest low nicotine and nicotine free	TPM (Cambridge method)	Dose-response observed	Chen, J 2008 (146)
XTT assay	Activity of mitochondrial dehydrogenases	CHO WBL strain	FTC	1R4F	TPM (Cambridge method)	Proliferation observed after1-6 h of exposure	Putnam, KP 2002 (44)

Abbreviations: FACS, fluorescence-activated cell sorting; HPAEC, human pulmonary artery endothelial cell.
*TPM or CSC indicated as reported in publication, but actual method shown in parenthesis.

Table 8. Proliferation assays for ST extracts

Assay	End point measured	Cell studied	Extraction solution and time	ST product	Results reported in study	Reference
BrdUrd incorporation	Proliferating cells incorporate BrdUrd label	Golden Syrian hamster oral epidermoid carcinoma cell line	DMEM	1S3	No significant differences observed	Mangipudy, RS 1999 (103)
		Human oral keratinocytes and fibroblasts	HBSS	Kentucky loose-leaf chewing tobacco, dry snuff, moist snuff	Proliferation of keratinocytes stimulated by low doses and suppressed by high doses, fibroblasts showed increased proliferation at all doses	Wang, Y 2001 (113)
Colony Formation Assay	Counting stained cells	CHO cells	H2O	Kentucky reference chewing tobacco	Concentration-dependent response observed	Yildiz, D 1999 (99)
^{3}H Thymidine uptake	Counting the number of cells that incorporate radioactive label	Embryonal mouse tongue epithelial cells	Dichloromethane	Snuff from Manglore	STE reduced proliferation	Gijare, PS 1989 (276)
		Spleen cells, T cells, and oral epithelial cells from rat	DMEM	Commercial Swedish moist snuff	Proliferation was inhibited by STE for all cell types, concentration-dependent response observed.	Hasseus, B 1997 (107)
		Human skin fibroblasts	DMEM	Commercial chewing tobacco	Dose- and time-dependent response observed.	Coppe, JP 2008 (105)
MTS	Reduction of tetrazolium salts to formazan dyes by cellular enzymes	Golden Syrian hamster oral epidermoid carcinoma cell line	DMEM	1S3	Concentration-dependent response observed, differs by exposure time	Mangipudy, RS 1999 (103)
MTT	Reduction of tetrazolium salts to formazan dyes by cellular enzymes	Normal human oral keratinocytes	PBS	Kentucky moist snuff	Concentration- and time-dependent response	Bagchi, M 2001 (144)
		Oral leukoplakia cells (AMOL-III)	H2O	Commercial khaini	Proliferation was observed at lower concentrations, cytotoxicity was observed at higher concentrations	Rohatgi, N 2005 (97)

thymidine is added to the culture medium and incorporated into newly synthesized DNA. After the labeling period, the unincorporated tritiated-thymidine is removed by washing and the remaining radioactivity is counted using a scintillation counter. For example, this method has been used to show a dose-dependent response in human cells (76, 150). Although this is a relatively simple and reliable method that is amenable to relatively high throughput screening, the use of radioactivity raises safety and waste disposal issues. These assays also can have important confounding factors when test substances affect the activity or expression of the enzymes involved in pyrimidine metabolism, which then impacts the degree to which the tritiated-thymidine is incorporated into newly synthesized DNA. A nonradioactive alternative involves the use of the thymidine analogue 5-bromo-2′-deoxy-uridine (BrdUrd). Highly specific antibodies that can recognize BrdUrd when incorporated into DNA are then used to identify cells that have synthesized new DNA during the labeling, either by immunostaining or flow cytometry. These assays also have been applied to TPM and CSC showing a dose-response effect (73, 75).

Cell Number–Based Proliferation Assays. Another way to infer the proliferation rate in a culture is to measure the change in cell number over time, or to compare the final cell numbers of different treatments. Although this is a widely used approach, it should be remembered that the number of cells in a culture is a function of both the cell division rate (proliferation) and the rate of cell death. This is particularly important to keep in mind when potentially toxic materials (such as from cigarette smoke) are being assayed for proliferative effects. Thus, it is important to evaluate the effects of materials at multiple concentrations and to evaluate the assays for concomitant cell death. Although there are methods for actually counting the numbers of cells in a culture, this can be tedious, and so most commonly used proliferation assays estimate the cell number by some indirect measures, e.g., the total amount of DNA or protein in the culture, or through some enzymatic assay of cell number.

One method commonly used to assess cell numbers in 96-well plates is the is the crystal violet assay, where the cells are fixed and stained with crystal violet dye, washed, and dried. The dye that diffused out of the cells is measured by absorbance at 540 nm. Dye uptake is proportional to the amount of cellular material on the plate and so gives a good estimate of cell number. Other popular assays make use of the same redox-activated tetrazolium compounds used for the assessment of cytotoxicity described above, e.g., WST-8. In this context, it is assumed that the level of dehydrogenase activity is the same in all cells and so total enzyme activity is a measure of cell number. This assumption, however, needs to be validated with appropriate controls because inhibitors of these enzymes within the test materials, or chemicals that have overlapping absorbance characteristics, could confound the interpretation of the assays. Using these assays, as shown in Table 7, several methods show the effects of cigarette smoke, including TPM, CSC, WS, and GVP on cell proliferation (21, 23, 44, 48, 52, 76, 83, 85, 146, 151, 152).

Cell Cycle Control. The cell cycle is tightly regulated through a coordinated series of checkpoints that are designed to ensure that the cell is ready to progress to the next phase, e.g., critical cell functions such as mitosis, cell replication, and detecting and repairing DNA damage. The cycle is regulated by cyclins and cyclin-dependent kinases, and other genes classified as tumor suppressor genes and proto-oncogenes that can either stimulate or inhibit the cell cycle. The loss of cell cycle control and attendant uncontrolled cell replication is a hallmark of cancer. Chemical insults that adversely affect cell cycle checkpoints can have profound effects on cell viability and the incorporation of DNA damage due to decreased DNA repair. Cell cycle assays provide information about the number of cells within the various phases of the cell cycle such as S phase (DNA synthesis), the function of the various checkpoints such as the G_1-S checkpoint (a pause to ensure that the cell is ready to enter the S phase), and the G_2-M checkpoint (a pause to ensure that the cell is ready to enter mitosis).

Cell cycle assays are typically conducted by harvesting treated cells and either fixing and permeabilizing them directly, or by isolating the nuclei from the cells followed by staining the DNA with a fluorescent dye and analysis by flow cytometry. The assay reports the number of cells in each phase of the cell cycle: G_1-G_0 with a DNA content of 2n, S phase with DNA content between 2n and 4n, and G_2-M phase with DNA content of 4n. The analysis of primary cell cultures is the most simple, but assays using cells that are aneuploid can be done by determining the DNA content and empirically determining the various phases of the cell cycle. As shown in Table 9, although CSC and TPM affect the cell cycle, the phases and check points can be affected differently among studies. Thus, due to differences in experimental conditions, there is little consistency. Whereas some tobacco studies show increases in the percentage of cells in G_0-G_1 phase and decreases in the number of cells in S phase (146, 153, 154), others show the reverse effect (21, 146, 155). Differences also may be due to the choice of cell culture. At present, it is unknown if TPM, CSC, GVP, or WS have different or similar effects on cell cycle in the same cell type. For PREP combustible products, it was reported that Eclipse cigarette TPM prepared by the FTC method had no effect on cell cycle control of normal human bronchial epithelial cells (155). However, both low-nicotine and nicotine-free Quest TPM/CSC altered the number of NHBE cells in different phases (146). There is only one study that we are aware of for STE on the cell cycle, which had an effect (114). There are no reports on cell cycle assay by PREP ST products.

Apoptosis Assays. Apoptosis, which is a form of programmed cell death, can be induced in response to a variety of stimuli including conflicting cellular signals, certain types of toxic insults, and through the activation of specific cell death receptors. Once initiated, programmed cell death involves a coordinated series of biochemical events that result in the sequential activation of lytic enzymes. These alterations can be observed with characteristic morphologic changes to the cell membrane, cell shrinkage, nuclear fragmentation, chromatin condensation, and chromosomal DNA fragmentation. Apoptosis, in contrast to necrosis (traumatic cell death), is a normal process that results in the remodeling of tissues as the body protects itself from cells that have accumulated

Table 9. Cell cycle assays by flow cytometry for cigarette smoke

Cigarette Studies								
Cell Model	Machine protocol	Cigarette type	Test substance*	Cell cycle effects as reported	G_0-G1 phase	S phase	G_2-M	Reference
Human normal breast epithelial cell line MCF10A	FTC	1R4F	CSC (Cambridge filter)	Dose-dependent response observed	Decreases compared with control at higher concentrations	Increase compared with control	Increase compared with control	Narayan, S 2004 (21)
Normal human bronchial epithelial cells	FTC	Kentucky 1R4F, Eclipse	TPM (Cambridge filter)	Dose-dependent response observed for 1R4F, but not for Eclipse	Decline for 1R4F, no change for Eclipse	Increase 1R4F, no change for Eclipse	Increase for 1R4F, no change for Eclipse	Fields, WR 2005 (155)
RAT-1 immortalized fibroblasts	ISO	N/A	TPM (glass fiber filter)	G_0-G_1 and S affected, but not G_2M	Increased	Decreased	Unchanged	Palozza, P 2005 (153)
RAT-1 immortalized fibroblasts	ISO	N/A	TPM (glass fiber filter)	G_0/ G_1 and S affected, but not G_2M	Increased	Decreased	Unchanged	Palozza, P 2006 (154)
Human lung epithelial cell line A549	N/A	Indian cigarette with filter	CSC (Solvent trap)	G_0-G_1 and S affected, but not G_2-M	Increased	Increased with lower CSC, decreased at highest concentration	Unchanged	Kaushik, G 2008 (76)
Normal human bronchial epithelial cells	FTC	Quest cigarette low nicotine, nicotine free and 2R4F	TPM (Cambridge filter)	Dose-dependent response observed f	Increased at 16 h, decreased with both products 48 h	Decreased at 16 h, Increased with both products 48 h	Decreased at 16 h, Increased with both products 48 h	Chen, J 2008 (146)
Human lung epithelial cells A549	N/A	Commercial filtered cigarette	AECS (Solvent trap)	Time- and dose-dependent inhibition of cell cycle progression observed	Sub-G_0-G_1 increased as G_0-G_1 decreased in a dose- and time-dependent manner	N/A	N/A	Das, A 2009 (152)

*TPM or CSC indicated as reported in publication, but actual method shown in parenthesis.

Table 10. Apoptosis testing for cigarette smoke

Assay	End point measured	Cell studied	Smoke conditions	Cigarette type	Test Substance*	Cytotoxicity reported in study?	Reference
Annexin V and PI measured by FACS	Measure apoptotic and necrotic cells	Human fetal lung fibroblasts (HFL-1 lung, diploid, human)	N/A	Unfiltered cigarettes	CSE (Solvent trap)	Dose-dependent increase	Carnevali, S 2003 (157)
		Human umbilical vein endothelial cells	N/A	Two commercial filtered cigarettes	CSE (Solvent trap)	Increase in cell death observed compared with control	Bernhard, D 2004 (277)
		Human lung epithelial cells A549	N/A	Commercial filtered cigarette	AECS (Solvent trap)	Dose-dependent increase	Das, A 2009 (152)
Annexin V assay kit	Annexin V binds to apoptotic cells	Primary airway epithelial cells, some transformed with SV40	MDPH	Commercial brands	CSC (Jet impaction), GVP (Solvent trap)	Dose-dependent increase	Hays, LE 2008 (162)
Annexin V/7-AAD flow cytometric assay	Annexin/7-AAD binds to apoptotic cells	Immortalized human oral keratinocytes cell line OKF6/TERT1	FTC	2R4F	CSE (Solvent trap)	Reduced cell death observed in chronic (6 mo in culture) CSE treatment	Chang, SS 2009 (158)
Caspase 3 Activation Assay	Measure active caspase 3	Immortalized human oral keratinocytes cell line OKF6/TERT1	FTC	2R4F	CSE (Solvent trap)	Cells treated for extended time periods were observed to have chronic Casepase-3 activation	Chang, SS 2009 (158)
		Human pulmonary artery endothelial cells	N/A	Long Life cigarettes	CSE (Cambridge method)	Dose-dependent increase	Hsu, CL 2009 (159)
		Human bronchial epithelial cell line BEAS-2B	FTC	2R4F	CSC (Cambridge method)	Dose-dependent increase	Carter, CA 2009 (160)
Elisa kit for nucleosome detection	Detection of free nucleosomes due to apoptosis	Normal human bronchial epithelial cells	FTC	Quest low nicotine, nicotine free, and 2R4F	CSC (Cambridge method)	Increased with higher dose for Quest nicotine-free but not low-nicotine or reference cigarette.	Chen, J 2008 (146)
FLICA Caspase detection kit	Stain-activated caspase 3 and 7	Mouse embryonic fibroblasts	N/A	2R1, 1R4F and four commercial cigarettes	MS (Solvent trap)	Dose-dependent increase	Lin, S 2009 (161)
Microscopy — Giemsa and Acridine orange	Morphologic changes associated with apoptosis	Rat, mice, human, and murine AMs, and human blood monocyte– derived macrophages	N/A	Commercial cigarette	CSE (Solvent trap), GVP (Solvent trap)	AMs exposed to CSE showed morphologic changes including cellular shrinkage and chromatin condensation	Aoshiba, K 2001 (156)
PI staining	PI stains necrotic cells	Human lung epithelial type-II cells A549	N/A	Indian cigarette with filter	CSC (Solvent trap)	Dose-dependent increase	Kaushik, G 2008 (76)
TUNEL assay dUTP labeling	Measure DNA fragments due to apoptosis	Rat, mice, human and murine AMs and human blood-monocyte derived macrophages	N/A	Commercial cigarette	CSE (Solvent trap), GVP (Solvent trap)	Dose- and time-dependent increase observed. GVP caused less apoptosis than CSE. All cells behaved similarly.	Aoshiba, K 2001 (156)
TUNEL staining	Measure DNA fragments due to apoptosis	Primary airway epithelial cells, some transformed with SV40	MDPH	Commercial brands	CSC (Jet impaction), GVP (Solvent trap)	Dose-dependent increase	Hays, LE 2008 (162)
		Rat-1 Fibroblasts	BAT protocol (ISO)	N/A	TPM (Glass fiber filter)	Dose-dependent increase	Palozza, P 2005 (153)

*TPM or CSC indicated as reported in publication, but actual method shown in parenthesis.

Table 11. Apoptosis assays used for ST extracts

Assay	End point measured	Cell studied	Extraction solution and time	ST product	Results reported in study	Reference
PI labeling analyzed by flow cytometry	PI stains DNA of damaged cells	Normal human oral keratinocytes	PBS	Reference smokeless chewing tobacco (2S1)	Concentration-dependent response observed	Bagchi, M 1999 (114)
		Golden Syrian hamster oral epidermoid carcinoma cells	DMEM	Reference smokeless moist tobacco (2S3)	Dose-dependent response observed	Mangipudy, RS 1999 (103)
		Golden Syrian hamster oral epidermoid carcinoma cells	DMEM	Reference ST (2S3)	Dose-dependent response observed	Banerjee, AG 2007 (100)
TUNEL with dUTP labeling	Measure DNA fragments due to apoptosis	Golden Syrian hamster oral epidermoid carcinoma cells	DMEM	Reference smokeless moist tobacco (2S3)	Dose-dependent response observed	Mangipudy, RS 1999 (103)
		Golden Syrian hamster oral epidermoid carcinoma cells	DMEM	Reference ST (2S3)	Dose-dependent response observed	Banerjee, AG 2007 (100)

genetic damage that cannot be repaired. Dysregulation of normal apoptotic control is frequently considered to be an important step in the carcinogenic process.

Assays for apoptotic cells typically take advantage of one or more of the characteristic biochemical changes that can distinguish them from cells undergoing necrotic cell death. However, the ability to make such a discrimination is highly time dependent, because cells at various points within the apoptotic process can share the physical characteristics of cells that are dying in other ways. Different apoptosis assays have optimal application in different contexts, depending on the design and timing of the assays. A common assay for the early stages of apoptosis takes advantage of the fact that the loss of membrane asymmetry is an early event that precedes loss of PM integrity, namely the flipping of phosphatidyleserine from the inner to the outer face of the PM. This event can be detected using fluorescent conjugates of the calcium-binding protein Annexin V that binds tightly and specifically to phosphatidyleserine. Cells to be assayed for apoptosis are harvested, stained with Annexin V and PI, and the staining is assessed by flow cytometry. Cells that are positive for Annexin V and negative for PI are deemed to be in the early stages of apoptosis. Other approaches that can be used involve assays that assess the activation of lytic enzymes such as caspases. This can be done using fluorescent substrate probes or through the use of antibodies specific to the cleaved form of the proteins. Other apoptosis assays, called DNA ladder assays, detect DNA fragments by gel electrophoresis, because apoptotic cells have DNA that are cleaved to 180 to 200 bp fragments by nucleases in a characteristic way.

Other assays include the terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) assay that involves the enzymatic labeling of the cleaved ends of the DNA and the Comet assay described below. It has been shown that treatment of cells with CSC or STE induces apoptosis, as indicated in Tables 10 and 11 (76, 100, 114, 146, 152, 153, 156-162). Only one study was found to measure apoptosis using a PREP. Chen et al. (146) reported that apoptosis was increased in the Quest nicotine-free cigarette compared with Quest low-nicotine or 2R4F reference cigarette. Few studies for STE have been conducted on apoptosis and the results showed dose-dependent increases with both reference STEs on normal human oral keratinocytes and golden Syrian hamster oral epidermoid carcinoma cells using the TUNEL assay (100, 103, 114). However, the results for commercial STE brands have not been published.

Genotoxicity Assays. Genetic damage is a principle component of carcinogenesis (although other mechanisms also are required). All cancers display a variety of genetic defects that range from individual base changes to gross chromosomal or clastogenic effects. Cancer cells accumulate DNA damage, even before there are morphologic changes to the cell; however, not all cells with genetic damage transform into cancer. Given that cancer is a disease that includes genetic damage, it is therefore reasonable that genotoxicity assays in *in vitro* systems are used as a screening method for a potential mutagenic effect in more complex biological systems, such as experimental animals and humans. Given that the presence of DNA damage does not necessarily indicate an effect on gene function or activation of a pathway that leads to cancer, and so direct extrapolation is not possible. There are different ways to assess DNA damage in cultured cells. Some genotoxicity methods detect gross chromosomal or clastogenic changes, such as CA (e.g., chromosomal breaks, gaps, and translocations), micronuclei, and SCE. Other methods include the detection of specific base mutations (i.e., insertions, deletions, transversions, and transitions).

Several assays will not be reviewed here, because they have not been widely applied to tobacco assessments, although they may be useful for this purpose. These include the hypoxanthine-guanine-phosphoribosyl-transferase test conducted in CHO or Chinese hamster lung V79 cells (163-165), a transgenic big blue mouse cell line assay (166, 167), a high throughput GreenScreen HC *GADD45a-GFP* assay using the human lyphoblastoid cell line TK6 (168, 169), and a yeast cell line that induces *RAD54*, a DNA repair gene, in response to mutagen exposure (170-172).

Chromosomal Aberration Assays. The detection of CAs is one of the most commonly used cytogenetic assays, in part because CAs are frequently observed in cancer. Also, there is data to indicate that increased CAs detected in humans might predict increased cancer risk (173-178), and CAs are increased in the blood cells of smokers (179, 180). DNA double strand breaks are the principal lesions in the process of CA formation, which are not induced directly, but follow other types of chromosomal damage and errors in DNA repair or synthesis (175). There are several types of CAs that can be detected, including chromosome breaks, gaps, acentric fragments, centric rings, and dicentrics (interchange between two separate chromosomes). These types of damage can be lethal to the cell. CAs also include translocations, but these usually are not assessed in tobacco *in vitro* toxicology tests. If not repaired properly, double strand breaks can lead to chromosome rearrangements, mutations, and oncogenic transformation (122, 175, 178). There is a classic methodology for detecting CAs that involves culturing cells with the test materials, e.g., TPM, CSC or STE, treating the cells with colcemid to arrest the cells in metaphase, staining the cells with giemsa, and then visually counting the CAs per cell (178, 181).

Typically, CHO cells are used, and sometimes human lymphocytes, but this can be done with essentially any rapidly proliferating cells. More recently, CAs have also been detected using other techniques, such as chromosome painting by fluorescent *in situ* hybridization (FISH; ref. 182). If translocations are of interest for assessing toxicity, then FISH methods can be used, which also will detect other specific gross chromosomal changes. One of the main advantages of FISH-based methods is that it is much easier for the nonexpert observer compared with more traditional CA detection techniques, but the FISH is more expensive and time consuming.

CA assays are generally more tedious than other assays for genotoxicity, such as those for detecting SCE and micronuclei (see below), and require special training and expertise on the part of the observer. It should be noted that some non–DNA-damaging agents can induce CAs, because they induce cytotoxicity or inhibit DNA synthesis, and so it has been suggested that the doses of agents used in such assays be limited to minimize cytotoxicity and decreases in the mitotic index (183, 184). Positive results are, however, sometimes only seen at high doses that decrease the mitotic index by >50%, and hence, as a screening test, it has been recommended that dose levels of test substrates reduce mitotic indices by 50% or more, although the false positive rate is increased (185). Whether this is an appropriate strategy for comparing tobacco products remains to be tested. Typically, false-positive rather than false-negative rates are preferred for non-tobacco screening of genotoxic agents for a qualitative assessment of possible human cancer risk, but for comparison of different tobacco products, this large decrease in high mitotic rates may not be optimal. Separately, it has been proposed that population doubling might be a more reliable criteria to use rather than mitotic index (186). The advantages and disadvantages of commonly used CA detection methods have been recently reviewed (174, 175, 178).

Table 12 summarizes available studies for CA induction by CSC and TPM. Cigarette smoke from reference and commercially available cigarette brands are well-documented to induce CAs (60, 62, 163, 165, 187). There are relatively few studies that compare CAs resulting from *in vitro* exposure to different types of conventional cigarettes, however, or by different smoking machine regimens, although differences in potency have been reported (62). PREPs also have received little attention, although it was reported that Eclipse cigarettes do not induce CAs in CHO cells (86). Very few studies of STE have been conducted for CAs, although tobacco plus lime and a Swedish moist snuff induce CAs in CHO cells (122, 188).

Micronucleus Assay. A micronucleus results when the spindle apparatus or DNA is damaged so that lagging chromosomal fragments form micronuclei, which can be visually observed and counted (19). The micronucleus assay has emerged as a commonly used method for assessing chromosome damage (19, 178, 189), because it is relatively inexpensive and quantifiable (178). The current methodology is based on the cytokinesis-block micronucleus assay, originally developed in 1985, in which cells are cultured and the dividing cells are identified by their binucleate appearance after treatment with a blocking agent such as cytochalasin B (189, 190). Recently, kits using automated methods have been developed for flow cytometric assays (131) and these methods may be preferred over manual scoring (28). The underlying mechanism, applicability, criteria for scoring, advantages, and disadvantages have been reviewed (19, 189, 191). As with other genotoxicity assays, there is some consideration that too little or too much cytotoxicity can yield more false-negative or positive results, and so the amount of cytotoxicity needs to be determined (185). In several *in vitro* experiments, cigarette smoke induced micronucleus in BALB/c-3T3 cell line, Chinese hamster lung V79 cells, Hepa1c1c7 cells, TAOc1BP(r)c1 cells, mouse lymphoma L5178Y/Tk+/− 3.7.2C cells, and others (Table 12; refs. 62, 77, 87, 192). A recent publication has indicated that the micronucleus assay using mouse lymphoma L5178/TK+/− cells was sufficiently quantitative to detect differences in DNA damage capacity for different types of commercial cigarettes with a 3-fold range in potency (62). There are some studies that also indicate that STEs can induce micronuclei (118, 193-195).

Sister Chromatid Exchange Assays. SCE occur when there is a symmetrical exchange of DNA segments between two sister chromatids of a duplicating chromosome. Given that identical DNA is exchanged, there is no known functional effect of this genotoxic damage. SCEs are formed during the S phase of the cell cycle and can be induced by chemicals that are S phase–dependent DNA-damaging agents (178). An SCE is formed from a delay in the spiralization pattern of the late replicating regions along the chromosome following DNA replication errors, but the molecular mechanism for this is unknown (178). The most commonly used SCE assay follows the original protocol of Perry and Wolff published in 1974 (196). Recent reviews for the application of SCE detection for different exposures and populations have been published (19, 176, 178). The method is based on exposing the test substance to a cell culture and then adding BrdUrd for incorporation into replicating DNA strands. The culture also is treated with colcemid to arrest cells

Table 12. Genotoxicity assays for CA, micronuclei, and SCE for cigarette smoke

Assay	Cell studied	Machine method	Cigarette
Sister chromatid exchange	CHO	FTC	European commercial cigarettes
	Cultured human lymphocytes	FTC	British commercial cigarette
	Cultured human lymphocytes	N/A	N/A
	CHO, cultured human lymphocytes	N/A	Commercial British cigarette
	Cultured human lymphocytes	ISO; Coresta no. 10	Commercial nonfilter cigarettes of American blend
	V79 Chinese hamster fibroblasts	N/A	N/A
	CHO-K1	FTC	An American blend cigarette
	CHO-WBL	FTC	Kentucky 1R4F
	V79 Chinese hamster lung cells	N	N/A
	CHO WBL	FTC	Kentucky 1R4F, commercial cigarettes and test cigarettes that heat tobacco with and without menthol
	CHO	FTC	1R4F, a menthol and a regular cigarette that heats tobacco
	CHO-WBL	FTC	Commercial and novel carbon filter cigarettes
	CHO-WBL	FTC	1R4F, 1R5F, cigarette that primarily heats tobacco (TOB-HT)
	CHO	FTC	Kentucky 1R4F and 1R5F, cigarette which primarily heats tobacco (TOB-HT)
	CHO	FTC	Commercial blend with and without honey
	CHO	FTC	Freon- or propane-expanded tobacco blend and control cigarettes
	CHO	FTC	Cigarettes containing increasing amounts expanded shredded tobacco stems and control cigarettes
	CHO	FTC	Commercial cigarettes with and without different amounts of fructose corn syrup
	CHO	FTC	Reference cigarette, test cigarettes with different levels of DAP, urea and cut rolled expanded stems
	CHO	FTC	Standard American tobacco blend, three test cigarettes with banded cigarette paper technologies and control cigarette
	CHO	FTC	Reference cigarette, commercial tobacco blend, with and without cast sheets
CA	CHO WBL	FTC	Kentucky 1R4F, commercial ultralow tar and menthol, cigarettes that heat tobacco with and without menthol
	CHO WBL	FTC	Kentucky 1R4F, commercial cigarettes and test cigarettes that heats tobacco with and without menthol
	CHO	FTC	1R4F, regular cigarette that heats tobacco, menthol cigarette that heats tobacco
	CHO	FTC	Kentucky 1R4F and 1R5F, cigarette that primarily heats tobacco (TOB-HT)
	CHO-K1	FTC and MDPH	2R4F, commercial cigarettes and cigarettes with single tobacco types
Micronuclei formation	BALB/c-3T3 cell line	N/A	A commercial filtered brand
	Chinese hamster lung V79 cells	N/A	Commercially available ultralow tar cigarettes
	Chinese hamster lung V79 cells	N/A	N/A
	Hepa1c1c7 cells and TAOc1BP(r)c1 cells	N/A	Kentucky 1R4F
	Mouse lymphoma L5178Y Tk+/− 3.7.2C cells	FTC and MDPH	Commercial cigarettes, reference cigarettes, and cigarettes with single tobacco types

*TPM or CSC indicated as reported in publication, but actual method shown in parenthesis.

in metaphase, which are then fixed and stained with giemsa. BrdUrd, which is taken up in place of thymidine, has weaker staining. This differential staining, detected after two cell replications, will allow for SCEs to be observed when a strand has both stained and unstained DNA. SCE detection is less laborious than detecting CAs, and similar to the micronucleus assay. Most studies use cultured CHO cells or human lymphocytes for the SCE assay, partly because it is a technical challenge to obtain a high percentage of synchronized metaphases using cell types that are slow to replicate. CSCs and TPM induce SCEs in *in vitro* studies and concentration-dependent responses have been observed (refs. 59, 67, 70, 84, 140, 197, 198; Table 12). However, this assay has had been rarely applied for the comparison of different tobacco products. For PREPs, SCEs were assessed for Eclipse cigarette TPM

Table 12. Genotoxicity assays for CA, micronuclei, and SCE for cigarette smoke (Cont'd)

Test material*	Reported Result	Reference
CSC (Cambridge filter)	Similar positive results for all cigarettes	De Raat, WK 1979 (198)
CSC	Dose-response reported for lymphocytes cultured from different smokers	Hopkin, JM 1980 (278)
CSC	Dose-response reported	Vijayalaxmi, 1982 (252)
CSC (N/A)	Dose-response reported for both cells, CSC caused more SCE in CHO than lymphocytes	Perry, PE 1983 (251)
CSC (Cold trap)	SCE frequency differed by fractionation of CSC	Curvall, M 1985 (66)
CSC (N/A)	Positive increase observed but no dose-response reported	Jongen, WM 1985 (164)
CSC (Cambridge method)	Dose-response reported	Rutten, AA 1986 (279)
TPM (Cambridge method)	Dose-response reported	Lee, CK 1989 (280)
CSC (Solvent trap)	Positive	Xing, SG 1989 (281)
CSC (Cambridge method)	Dose-response increase in SCE observed in reference and commercial cigarettes, but not for test cigarettes that heat tobacco	Doolittle, DJ 1990 (163)
MWS (Cambridge method), GVP (Solvent trap)	Dose-dependent increases observed in 1R4F, neither regular or menthol cigarettes which heat tobacco increased SCE frequency	Lee, CK 1990 (282)
TPM (Cambridge method) and WS (Exposure chamber)	Dose-response reported for CSC but no difference between commercial and novel carbon filter cigarettes, WS of novel carbon cigarettes induced fewer SCE	Bombick, DW 1997 (59)
WS	Reference cigarettes had higher SCE than TOB-HT on a per cigarette basis	Bombick, DW 1998 (86)
CSC (Cambridge method)	Dose-response reported for 1R4F and 1R5F, but not TOB-HT	Bombick, BR 1998 (145)
TPM (Cambridge method)	No statistically significant differences in were observed between test cigarettes and reference cigarettes	Stavanja, MS 2003 (225)
CSC (Cambridge method)	Dose-dependent increase observed, no differences were detected between test and control cigarettes	Theophilus, EH 2003 (70)
CSC (Cambridge method)	Dose-dependent increase observed, no differences were detected between test and control cigarettes	Theophilus, EH 2004 (67)
TPM (Cambridge filter)	Dose-response reported, no difference with fructose	Stavanja, MS 2006 (140)
CSC (Cambridge method)	Dose-response increase observed, no difference between reference and test CSC	Stavanja, MS 2008 (197)
CSC (Cambridge method) and WS (Exposure chamber)	No significant difference between control and test cigarettes in both CSC and WS	Theophilus, EH 2007 (68)
TPM (Cambridge Method) and WS (Exposure chamber)	Concentration-dependent increase observed with both CSC and WS.	Potts, RJ 2009 (84)
CSC (Cambridge method)	Dose-response reported for reference and commercial cigarettes, but not for the heating cigarettes	Doolittle, DJ 1990 (163)
SSCS (Cambridge method)	Dose-dependent increases in CAs observed for 1R4F and commercial cigarettes but not for test cigarettes	Doolittle, DJ 1990 (165)
MWS (Cambridge method), GVP (Solvent trap)	Dose-dependent increases observed in 1R4F, neither regular or menthol cigarettes that heats tobacco increased aberration frequency	Lee, CK 1990 (282)
CSC (Cambridge method)	Dose-response reported for reference cigarettes but not for TOB-HT	Bombick, BR 1997 (60)
CSC (Cambridge method)	Dose-response reported for all cigarettes except 100% burley tobacco.	DeMarini, DM 2008 (62)
CSC (Solvent trap)	Dose-response reported at lower concentrations, highest concentration toxicity was observed.	GU, ZW 1992 (192)
CSC (Solvent trap)	Dose-response reported for both kinetochore-positive and kinetochore-negative micronucleus.	Channaraya, P 1992 (77)
CSC (Cambridge filter), WS and GVP (Exposure chamber)	Dose-dependent response observed, WS increased micronuclei formation more than GVP	Massey, E 1998 (87)
CSC (Glass fiber filter)	Dose-response reported, but more in Hepa cells	Dertinger, SD 1998 (283)
CSC (Cambridge method)	All CSCs responded positively at the top concentration, MDPH smoking condition produced similar results to FTC	DeMarini, DM 2008 (62)

and WS, and reportedly the TPM generated using the FTC method did not induce SCEs (59). For STEs, only limited studies have been applied for inducing SCE *in vitro*, but positive studies are available, including for snus (122, 188, 199).

Comet Assay. The Comet assay, otherwise known as the single-cell gel electrophoresis assay, detects single and double strand chromosomal breaks by assessing DNA size and fragmentation. Given that DNA fragmentation also is a feature of apoptosis and other types of cell death, interpretation of the data depends heavily on study design and the timing of exposures. The assay involves casting the exposed cells in a gel that is then treated with solutions to induce cell lysis. The gel is then exposed to an electrical field in a buffer system such that fragmented DNA migrates out of the nucleus and forms a cometlike tail, with small DNA fragments migrating faster than larger ones. The tail can then be visualized by staining

Table 13. *Salmonella* strain specificity*

	Compounds	TA98	TA100
Sequence specificity		Frameshift mutation	Missense mutation
Special characteristics		Parent strain TA1538, with pKM101 plasmid, ampicillin resistant	Parent strain TA1535 with pKM101 plasmid, ampicillin resistant
BaP	Benzo(*a*)pyrene	Positive (+/−)	Positive (+/−)
Trace metals	Cadmium	Negative	Negative
	Lead	Negative	Negative
	Nickel	Equivocal	Equivocal
	Chromium	Positive (+/−)	Positive (+/−)
Carbonyls	Formaldehyde	Positive (+)	Positive (+)
	Acetaldehyde	Negative	Negative
	Acetaldehyde oxime	Positive (+/−)	Positive (+/−)
	Crotonaldehyde	Positive (+/−)	Positive (+/−)
Humectants	Glycerol	Equivocal	Equivocal
TSNAs	*N*'-nitrosoanabasine (NAB)	N/A	N/A
	4-(Methylnitrosamino)-1-(3-pyridyl)-1-butanol (NNAL/NNA)	N/A	N/A
	N'-nitrosonornicotine (NNN)	N/A	Reactive (285)
	4-(*N*-methyl-*N*-nirtosamino)-1-(3-pyridyl)-1-butanone (NNK)	N/A	N/A
	N'-nitrosoanabatine (NAT)	N/A	N/A
Volatile nitrosamines	*N*'-nitrosodimethylamine (NDMA)	Positive (+)	Positive (+/−)
	N-nitrosopyrrolidine (NPY)	N/A	Positive (287)
	N-nitrosodiethanolamine (NDELA)	Positive (+)	Positive (+)
	NDEA (289)	Positive	Positive (+/−)
	NMOR (290)	Positive (+)	Positive (+/−)
	Nitrosopiperidine (NPIP)	Positive (−)	Positive (+/−)
	Nitrososarcosine (NSAR)	N/A	N/A
PAHs	Phenanthrene	N/A	N/A
	Pyrene	Positive (+/−)	Positive (+/−)
	Napthalene	N/A	N/A
	1-Methylphenanthrene	Positive (+)	Positive (+/−)
	Benzo[k]fluoranthene	Positive (+/−)	Positive (+/−)
	Benzo[b]fluoranthene	Positive (+/−)	Positive (+/−)
	Chrysene	N/A	N/A
	Dibenz(a,h)anthracene	Positive (+/−)	Positive (+)
Aromatic Amines	O-Toluidine	Positive (+/−)	Positive (+)
	1-Naphthylamine 2-Aminonapthlene 3-Aminobiphenyl	Positive (+/−)	Positive (+/−)
	4-Aminobiphenyl	Positive (+/−)	Positive (+)
	O-Anisidine	Positive (+/−)	Positive (+/−)
ROS			
Other Chemicals	Nitrate	Negative	N/A
	3-(Methylnitrosamino)-propionic acid (MNPA)	N/A	N/A
	4-(Methylnitrosamino)-butyric acid (MNBA)	N/A	N/A
	Ammonia	Negative	Negative
	Anthracene	N/A	N/A
	Arsenic	N/A	N/A
	Urethane	Positive (+/−)	Positive (+/−)

* All compounds marked as positive, negative or equivocal are listed as such by the National Toxicology Program (http://ntp-apps.niehs.nih.gov/ntp_tox). Plus and minus symbols following all positive results indicate the following: (+/-) positive with and without S9, (+) only positive with S9, (-) positive without S9 activation. Compounds marked as N/A were not listed in the NTP database.
Abbreviations: ROS, reactive oxygen species; TSNA, Tobacco-Specific Nitrosamines; PAH, polycyclic aromatic hydrocarbon.

the gel with ethidium bromide and observation under UV illumination. The precise conditions for cell lysis and subsequent electrophoresis steps are chosen based on what type of DNA damage needs to be detected. A consensus decision made by an international workshop on the Comet assay was that the alkaline (pH >13) version of the assay is the methodology of choice for assessing genotoxicity in general (200). Because the Comet assay is sensitive to cytotoxicity, it is recommended that this also be assessed (28). There is some consideration that too little or too much cytotoxicity can yield more false negative or positive results (185). There are automated methods to score the comets, and different ways to report the scores are acceptable (28). The newer automated methods allow for high throughput and better reproducibility.

TPM has been show to increase DNA damage measured by the Comet assay in a dose-dependent fashion (62, 146, 201, 202). Using the Comet assay, STE from a commercial snuff product was also reported to induce concentration-dependent DNA damage in normal human fibroblasts (105). Another model that can be assessed for genotoxicity by Comet formation uses either cells or the

Table 13. *Salmonella* strain specificity (Cont'd)

TA1535	TA1537	TA102	YG1024
Missense mutation Ampicillin resistant	Frameshift mutation Ampicillin resistant	Missense mutation pKM101 and pAQ1 plasmids, tetracycline and ampicillin resistant Detects DNA cross-linking agents, e.g., formaldehyde and acrolein and ROS	Frameshift mutation Parent strain TA1538, with pKM101 plasmid, ampicillin resistant O-acetyl transferase-overproducing strain
Positive (+/−)	Positive (+/−)	N/A	N/A
Negative	Negative	N/A	N/A
Negative	Negative	N/A	N/A
Negative		N/A	N/A
Positive (+/−)		N/A	N/A
Positive (+/−)	Positive (+/−)	Positive (+/−)	N/A
Negative	Negative	N/A	N/A
Positive (+)		N/A	N/A
Positive (+/−)	Positive (+/−)	N/A	N/A
Negative	Equivocal	N/A	N/A
N/A		N/A	
Reactive (284)		N/A	
Reactive (286)		N/A	
Reactive (286)		N/A	
N/A		N/A	
Positive (+/−)/Reactive (286)	Positive (+/−)	N/A	
Reactive (288)		N/A	
Positive (+/−)		N/A	
Reactive (289)		N/A	
Reactive (290)		N/A	
Positive (+)	Positive (+/−)	N/A	
N/A		N/A	
N/A		N/A	
Positive (+/−)	Positive (+/−)	N/A	
N/A	Negative	N/A	
N/A		N/A	
N/A		N/A	
N/A		N/A	
N/A		N/A	
N/A		N/A	
Positive (+/−)		N/A	Reactive (220, 221, 291)
N/A		N/A	
		N/A	
		N/A	
Positive (+/−)	Positive (+/−)	N/A	
Negative	Negative	N/A	
		Reactive (292)	N/A
N/A		N/A	N/A
N/A		N/A	N/A
N/A		N/A	N/A
Negative		N/A	N/A
N/A		N/A	N/A
N/A		N/A	N/A
Positive (+)	Positive (+/−)	N/A	N/A

whole insect of *Drosophila melanogaster*, but whether this nonmammalian system leads to an improvement over other cell systems for tobacco has not been tested (203).

Mouse Lymphoma Assay. The mouse lymphoma thymidine kinase (TK) assay (MLA) is another widely used *in vitro* mutation assay that has an advantage because it relies on mammalian cells and detects a wide range of genetic modifications (i.e., point mutations, larger scale chromosomal changes, recombination, and others; refs. 204, 205). Thus, another advantage is that it reports the effects of mutagens that cause both point mutations and clastogenic effects. The assay uses the L5178Y mouse lymphoma cell line that is genetically engineered to only have one TK gene. It is relatively easy to perform, is sensitive, and has been used for many different mutagens (205-207). The assay is based on a treatment with a lethal chemical metabolic substrate for TK, namely trifluorothymidine. TK is not an essential enzyme, and the mutation of the TK gene through the genotoxic actions of the test materials resulting in its inactivation prevents the metabolism of the triflourothymidine, thereby allowing the cells to survive in the presence of trifluorothymidine. Using this test, the mutagenic activity of TPM prepared from an EHC smoking system was lower than that from the other conventional cigarettes (205). There are many factors that can affect the performance of this assay, including the time between exposure and mutation formation and cell density,

and as with other genotoxicity assays, the MLA may also give false positive results (208).

Ames Mutagenicity. The most commonly used method for the detection of mutagenicity is the Ames Test, named for Bruce Ames, and also called the Salmonella histidine reversion assay (209). This assay was designed to detect a wide range of chemical substances that can produce genetic damage that leads to gene mutations (210), including for complex environmental and biological mixtures (209). The Ames test is inexpensive, rapid, and easy to perform. It is based on inducing and then detecting DNA base mutations, deletions, and insertions that can be the cause of many human genetic diseases. The Ames test uses Salmonella typhimurium bacteria that have a genetic defect in one of the genes responsible for histidine synthesis, such that the cells cannot survive without histidine supplementation in their culture media (209). Each strain is designed to be responsive to mutagens that act via a different mechanism, i.e., the induction of frame shift or bp mutations. When the Salmonella bacteria are exposed to a mutagen, new mutations at or near the original histidine site can occur, restoring the gene's function and allowing the cell to grow normally and form colonies. Hence, the mutation reverts the cell to a phenotype that no longer requires exogenous histidine. The revertant colonies are counted and compared with those found in positive and negative controls. Guidelines for conducting the assay are available.[13] The number of revertant colonies per plate is increased by exposure to mutagenic agents, usually in a dose-dependent manner (210). Additional cell strains have been developed to enhance the sensitivity for a wide variety of substances. For example, the (rfa) mutation has increased permeability to large molecules such as benzo(*a*)pyrene; the uvrB mutation that causes a break in a gene coding for DNA excision repair and causes the bacteria to be biotin-dependent; and the addition of the pKM101 plasmid that enhances error-prone DNA repair (209). The use of appropriate controls is critical because several of the strains commonly used have a significant spontaneous reversion rate, which also can change over time. Databases for reporting Ames test and other genotoxicity assay results of chemicals with different physico-chemical properties show that many chemicals that are positive in the Ames test also exhibit mutagenicity in other tests.

The Ames assay routinely incorporates the use of rodent liver fractions to allow for metabolic activation of test substances, because most mutagens require metabolic activation before they can damage DNA (211). The rodent liver fractions are called S9, because this is the fraction of liver homogenates that contain the metabolic enzymes.

Ames data, reported as revertants per some unit measure, can be analyzed in different ways to determine if there is a positive result (212). In one method, a simple doubling of the number of revertants can be considered positive, although this is an arbitrary cutoff and many have questioned this method (212-214). The two-fold rule can be changed to a three-fold rule for strains that have high mutagenic background (212). Alternatively, the slope of the linear portion of the dose-response curve can be determined and reported as revertants per some unit measure (e.g., per milligram of tar), also known as the specific activity (215). Relative potencies of different products can be assessed by using the method of Margolin and Kaplan that assesses competing effects on mutagenicity and cytotoxicity (216). This method also incorporates an assessment of plate-to-plate variability, and so laboratory variation is considered. The most commonly used method is the two-fold rule, and then the method for assessing slopes of the linear region, although the two-fold rule is considered less than ideal (212). Various software are available to analyze Ames data, including in the public domain (212).

The choice of the optimal *Salmonella* strain for Ames testing is determined by the test substance, as different strains have different sensitivities and specificities. Table 13 provides a summary of some available *Salmonella* strains and their sensitivities, although other strains are available (82, 210). Whether one strain is more sensitive to others has not been systematically determined and so currently the only useful information is a yes/no designation. For TPM and CSC, the strain TA98 is frequently used for primary screening because it is sensitive to the basic and neutral fractions, which contain the heterocyclic amines and aromatic amines that are the primary source of mutagenicity in TPM and CSC (62). However, because of the added sensitivities of TA100, it is frequently used in addition to TA98, and is sometimes better able to distinguish different products (127). For STE, TA100 has shown more mutagenicity than TA98 (124, 217).

Newer and more reactive strains have been developed to add additional information for the assessment of cigarette smoke in the Ames assay, namely the YG1024, YG1029, YG1041, and YG1042 strains (218-220). An *Escherichia coli* strain also has been developed, because this strain has an AT bp at the primary reversion site, but a deficient excision repair system. Strains TA102, *E. coli* WP2, and *E. coli* WP2(pKM101) are known to detect certain oxidizing mutagens, cross-linking agents, and hydrazines. When the nature of the materials being tested is unknown, a battery of strains should be tested, as will be the case for complex mixtures generated from newly developed tobacco products.

The Organization for Economic and Cooperative Development[14] has issued guidelines calling for at least five strains of bacteria to be used.[15] These should include at least four strains of *S. typhimurium* (TA1535, TA1537 or TA97a or TA97, TA98, and TA100). However, these strains may not detect certain oxidizing mutagens, cross-linking agents, and hydrazines, and so either *E.coli* WP2 strains or *S. typhimurium* TA102 are included. Alternatively newer YG strains might be used that include plasmids carrying a nitroreductase gene (YG1021 and YG1026) sensitive to nitroarenes or an O-acetyltransferase gene (YG1024 and YG1029) sensitive to aromatic amines (210, 219-221).[16]

Ames assays have been widely applied to assess the mutagenicity of TPM, CSC, WS, and GVP from cigarettes

[13] http://www.oecd.org/dataoecd/18/31/1948418.pdf

[14] http://tobaccodocuments.org/rjr/508352445-2461.html

[15] http://www.mged.org

[16] http://tobaccodocuments.org/rjr/508352445-2461.html; http://www2.tobaccodocuments.org/pm/2023949728-9741.pdf; http://www2.tobaccodocuments.org/pm/2501133413-3424.pdf; http://legacy.library.ucsf.edu/tid/mxa35d00

with varying tar yields and cigarette designs, with many positive results (Table 14). The Ames assay has been useful for identifying unpredicted paradoxical effects. For example, there is substantial data from internal tobacco company documents to show that increasing filter ventilation, which decreases tar yields, actually increases Ames mutagenicity on a per milligram of tar basis.[17] There are several possible reasons for this, including longer burn time for the tobacco.[17] In a published study, there were more revertants per milligram tar and TPM, but less in a per cigarette basis, for TA100 comparing Marlboro Lights to Regulars, and the results for TA98 trended in the same directions (127). The Ames test also has been used to identify the effects of various changes in tobacco constituents that vary nitrogen concentrations, where low-nitrogen cigarettes with a carbon filter are reported to generate extracts with reduced mutagenicity compared with a commercial blend with a carbon filter (59). For low-nicotine cigarettes, reducing nicotine had no effect on Ames mutagenicity (146). Published studies from tobacco company laboratories indicate that flavorings and casing ingredients do not affect mutagenicity (72, 78, 132, 222, 223), although other internal company documents indicate that others can and these generally have not been incorporated into market products. WS injected over the assay plates were able to mutate TA98 and TA100, in the presence of S9, but the vapor phase was only able to induce a mutagenic signal in TA100, YG1029, YG1042, and the *E. coli* strain WP2uvrApKM101 in the absence of S9 mix (82).

The impact of smoking machine protocols on the mutagenic potential of cigarettes has also been studied. Roemer et al. (139) reported that when the same cigarette was smoked with the FTC and MDPH method, the more intense MDPH protocol resulted in condensates that were four times more mutagenic. DeMarini et al. (62) reported similar results. However, Rickert, et al. (141) reported that although intensive smoking HC conditions compared with FTC conditions gave higher TPM yields on a per cigarette basis, the mutagenicity reduced when the results were reported on a per unit TPM basis. This was likely due to the reduced ventilation when 100% of the holes are blocked for the HC method.

The Ames test has been used to investigate the mutagenicity of some PREPS. The Premiere cigarette, developed in the 1980s, reportedly did not increase the number of revertants in four different *salmonella* strains compared with K1R4F, ultralight tar cigarettes and ultralight tar-menthol cigarettes (163, 165). Eclipse cigarettes, which purportedly heat tobacco instead of burning it, when smoked under FTC conditions, were reported to have reduced mutagenicity compared with conventional low-tar burning cigarettes (61). Experiments comparing the mutagenicity of EHC against other types of cigarettes reported that these had lower mutagenicity than Marlboro lights and Marlboro Ultra Lights (129), and that two prototypes of EHCs were 90% less mutagenic than conventional cigarettes (224). Recent studies have indicated that TPM from Quest low-nicotine cigarettes and nicotine-free cigarettes showed no statistical difference compared with the 2R4F reference cigarette (146).

ST products variably show genotoxicity in the Ames assay. Early tests showed positive results in TA98 and TA100 with extracts of chewing tobacco, but only after treatment with nitrite under acidic conditions (119, 225) and TA102 (119). Aqueous extracts of five ST products were positive with TA100, and based on that study, it seemed that TA98 was not sufficiently sensitive for ST evaluations (124). However, in another study, both the aqueous and methylene chloride extracts of Swedish snuff was tested, and only the methylene chloride extract was found to be mildly mutagenic in TA98, TA100, and TA1537 (122). More recently, pouched and loose wet snuff from typical U.S. brands, tobacco tablets, loose dry snuff made with low-TSNA tobacco, and two types of ST products from India (gutkha and zarda) were tested in the Ames assay using a DMSO extraction method (217). The dose response curves for tests with TA100 with S9 activation had variable slopes that were statistically significantly different depending on the analysis method; but even for TA100, none met the two-fold rule, and there was no response for TA98. Thus, it is unclear what the best methods are to assess STE.

Epigenetic Assays. Although most toxicologic effects involving cancer pathways that are studied focus on mutagenic and clastogenic end points, nongenotoxic mechanisms also can be studied. The most frequently used method assesses gap junction intercellular communication (GJIC). GJIC normally allows direct exchange of small water soluble molecules and ions between the cytoplasm of one cell and that of its neighbors; GJIC plays an important role in the regulation of cell growth, differentiation, oncogenic transformation, hormone secretion, and electrical coupling (226). With increased inhibition, it is considered by analogy to reflect tumor promotion mechanisms in experimental animal tumor initiation-promotion studies. Gap junctions are composed of a family of transmembrane proteins called the connexins, and the alignment of two compatible hemichannels forms the complete gap junction channel between two adjacent cells. Each hemichannel is a hexamer of connexins, composed by either homomers or heteromers (226). Connexins are suggested to be a family of tumor suppressor genes and most tumor cells have a reduced GJIC ability, suggesting the importance of intact GJIC in growth and differentiation control. Gap junction functionality can be assessed *in vitro* by direct measurement of the transfer of small molecules from one cell to another, for example, the transfer of fluorescent dyes between cells. Dye transfer can then be directly visualized and measured by microscopy, although flow cytometry methods are increasingly being used (226). This method has the advantage that a large cell population can be analyzed rapidly, has higher sensitivity, and the assay can evaluate the communication between the same or different cell types. In this assay, donor cells are labeled with Calcein AM, and recipient cells labeled with another dye (that is not subject to transfer through gap junctions) or not labeled. Donor cells are then parachuted on top of the recipient cells and dye transfer can be detected by flow cytometry (226). Although through-put is increased with flow cytometry, this assay overall is not particularly amenable to high throughput analysis.

[17] http://tobaccodocuments.org/rjr/508352445-2461.html; http://www2.tobaccodocuments.org/pm/2023949728-9741.pdf; http://www2.tobaccodocuments.org/pm/2501133413-3424.pdf

Table 14. Summary of Ames test done with cigarette smoke

Strains	Smoking conditions	Cigarettes
TA98 and TA100	FTC	European commercial cigarettes
TA98 and TA100	ISO; Corresta no. 10	Commercial nonfilter cigarettes of American blend
TA100 and TA1538	FTC	16 low-tar cigarettes from 1-10 mg tar and a high-tar cigarette
TA98, TA100, TA1535, TA1537, and TA1538	FTC or until complete consumption of heat source	1R4F reference cigarette, commercial cigarettes and cigarettes that heat but do not burn
TA98 and TA100	FTC	1R4F, regular cigarette that heat tobacco, menthol cigarette that heat tobacco
TA98 and TA100	FTC	K1R4F and 73 brand styles of U.S. market cigarettes
TA98, TA100, YG1021, YG1024, YG1026, YG1029, TA98NR, TA100-DNP$_6$, TA98-1,8-DNP$_6$, and TA100NR	N/A	Commercial cigarettes
TA98, TA100, and TA1538	IARC	2R1
TA98, TA100, TA1535, TA1537, and TA1538	FTC	1R4F, 1R5F, and a cigarette that heats tobacco (TOB-HT)
TA98	FTC	Commercial cigarettes and cigarettes with a novel carbon filter with varying nitrogen contents
TA98 and TA100	FTC	K1R4F Kentucky Reference Cigarette
TA98 and TA100	FTC	Cigarettes of 100% Flue cured or 100% burley tobacco with and without treatment for protein removal
TA98 and TA100	FTC	K1R4F, K1R5F and commercial cigarettes with different tar yields
TA98 and TA100	Different temperatures	0.25 g tobacco tablets
TA98, TA100, TA102, TA1535, and TA1537	ISO	K1R4F, cigarettes with different casing materials, flavorings and tobacco, including menthol
TA98 and TA100	FTC	Standard commercial blend with and without honey
TA98 and TA100	FTC	Freon or propane expanded tobacco blend and reference cigarettes
TA98, TA100, TA102, TA1535 and TA1537	ISO	Typical U.S. blends with different casings and flavoring
TA98	FTC, FTC intense (50/30/2), HC and MDPH	Eclipse and commercial ultralights
TA98 and TA100	FTC	Cigarettes containing increasing amounts expanded shredded tobacco stems
TA98, TA100, TA1535, TA1537, and TA102	ISO	1R4F or 2R4F, test cigarettes containing typical commercial tobacco blend no additives with cellulose acetate filters
TA98, TA100, TA1535, TA1537, and TA102	ISO	1R4F or 2R4F, test cigarettes containing typical commercial tobacco blend no additives with cellulose acetate filters
TA98 and TA100	FTC	Commercial cigarettes and test cigarettes with different fructose corn syrup amounts
TA98, TA100, and TA1537	FTC	1R4F and 2R4F
TA98 and TA100	ISO (modified for cigars, bidis, and pipe tobacco)	2R4F, five commercial cigarettes, two brands of cigars, two brands of cigarillos, two brands of bidis, and two brands of pipe tobacco
TA98 and TA100	ISO	2R4F
TA98 and TA100	ISO and HCI	K1R4F, K1R5F, and Canadian Monitor
TA98 and TA100	FTC	Standard American tobacco blend, three test cigarettes with banded cigarette paper technologies and control cigarette
TA1535, TA1537, TA1538, TA98, TA100, TA102, WP2uvrApKM101, YG1026, YG1029, YG1042	ISO	K2R4F and commercially available light and ultralight cigarettes

(*Continued on the following page*)

Table 14. Summary of Ames test done with cigarette smoke (Cont'd)

Analyte*	Results	Reference
CSC (Cambridge method)	S9 treated with Aroclor 1254 mutagenic in both strains.	De Ratt, WK 1979 (198)
CSC (Cold trap)	None of the fractions induced an increase in revertants.	Curvall, M 1985(66)
WS (Solvent trap)	WS from light tar cigarettes were less mutagenic compared with high tar cigarettes on a revertant/milligram basis.	Chortyk, OT 1990 (293)
TPM (Cambridge method)	CSC from 1R4F, ULT and ULT-menthol: concentration-dependent increase in revertant number with TA98, TA100, TA1537, and TA1538 CSC from TEST cigarettes did not cause an increase in the number of revertants	Doolittle, DJ 1990 (163)
MWS (Cambridge method)	1R4F induced dose-dependent mutagenicity, neither regular or menthol cigarettes that heat tobacco were mutagenic in TA98 or TA100	Lee, CK 1990 (282)
TPM (Cambridge method)	No significant differences between the mutagenicity of U.S. market brand cigarettes and K1R4F	Steele, RH 1995 (294)
CSC (Solvent trap)	All strains showed dose-dependent response with S9. Strains were ranked according to mutagenic response.	De Flora, S 1995 (291)
MS and CSC (DCM-eluted glass fiber filter)	Dose-response observed. Probe hybridization showed different frequencies of mutational events. Two or three freeze thaw cycles did not significantly alter mutagenicity.	DeMarini, DM 1995 (295)
CSC (Cambridge method)	1R4F induced concentration-dependent increases in all strains except TA1535. 1R5F induced concentration-dependent increases in TA98, TA100, TA1538. TOB-HT negative.	Bombick, BR 1997 (60)
CSC (Cambridge method)	Tobacco nitrogen content modified the mutagenicity more than filter type	Bombick, DW 1997 (59)
CSC (Cambridge method)	CSC was mutagenic in both TA98 and TA100	Putnam, KP 1999 (43)
TPM (Cambridge method)	Reduced mutagenicity was observed in cigarettes treated for protein removal in both strains	Clapp, WL 1999
CSC (Cambridge method)	No statistical differences between full flavor low tar and 1R4F. Ultra low tar more mutagenic than 1R5F on a revertant/cigarette basis.	Chepiga, TA 2000 (296)
N/A	Mutagenicity increases as temperature increases from 400-550°C	White, JL 2001 (297)
CSC (Glassimpaction trap)	Dose-response increase observed with TA98, TA100 and TA1537, and TA98 and TA100, there were no statistically significant differences among the test cigarettes	Roemer, E 2002 (72)
TPM (Cambridge method)	No statistically significant differences in mutagenicity were detected between test cigarettes	Stavanja, MS 2003 (223)
CSC (Cambridge method)	Dose-dependent increase observed, no differences were detected between test and reference cigarettes	Theophilus, EH 2003 (70)
CSC (Cambridge method)	Addition of ingredients to test cigarettes did not increase the mutagenic activity	Baker, RR 2004 (78)
CSC (Cambridge method)	Mutagenicity of Eclipse CSC was statistically lower than the other cigarettes for almost all comparisons	Foy, JW 2004 (61)
CSC (Cambridge method)	Dose-dependent increase observed, no differences were detected between test and reference cigarettes	Theophilus, EH 2004 (67)
CSC (Glass impaction)	No significant increase in mutagenicity in licorice treated cigarettes compared with control cigarettes	Carmines, EL 2005 (132)
CSC (Glass impaction)	No significant increase in mutagenicity in glycerin treated cigarettes compared with control cigarettes	Carmines, EL 2005 (131)
CSC (Cambridge method)	Dose response increase in mutagenicity observed, no difference with fructose	Stavanja, MS 2006 (140)
TPM (Cambridge method)	No significant differences between 2R4F and 1R4F with TA100 and TA1537, 2R4F mutagenicity was lower than 1R4F by revertant/milligram tar basis in TA98	Counts, ME 2006 (130)
TPM (Cambridge method)	Concentration-dependent response observed. Air-cured products showed higher mutagenicity on a revertant/milligram nicotine basis.	Rickert, WS 2007 (217)
WS and GVP (Exposure chamber)	Dose-dependent increase observed, highest response observed with 30% S9 activation. Flow rate and dilution rate influenced mutagenicity.	Aufderheide, M 2007 (298)
TPM (Cambridge method), CSC (Electrostatic collection method)	No significant effect on TPM activity between CFP method and ESP method. Specific activity of TPM prepared under ISO is greater than that prepared under HCI	Rickert, WS 2007 (141)
CSC (Cambridge method)	Slopes of control and test cigarettes were similar	Theophilus, EH 2007 (68)
WS and GVP	Dose response reported for WS and GVP	Aufderheide, M 2008 (82)

(*Continued on the following page*)

Table 14. Summary of Ames test done with cigarette smoke (Cont'd)

Strains	Smoking conditions	Cigarettes
TA98 and TA100	FTC	Quest low-nicotine and nicotine-free cigarettes and 2R4F
TA98, TA100, TA1535, TA1537, and TA102	ISO	Research cigarettes consistent with American tobacco blends with and without potassium sorbate, 2R4F
TA98 and TA1041	FTC, MDPH	2R4F, six commercial, and three experimental cigarettes with single tobacco types
TA98, TA100, TA102,TA1537, and TA1535	FTC/ISO, MDPH, HPP	Two EHC prototypes, eight commercial cigarettes, and K1R4F
TA98, TA100, TA102, TA1535, TA1537	ISO	1R4F, three commercial cigarettes
TA98 and TA100	FTC	Reference cigarette, test cigarettes with different levels of DAP, urea and cut rolled expanded stems
TA98, TA100, TA102, TA1535, and TA1537	ISO, HPP	Two Marlboro brands, 2R4F, and EHC
TA98 and TA100	FTC	Reference cigarette, commercial tobacco blend, with and without cast sheets
TA98 & TA100	FTC with modifications for tar production	1R5F, 2R4F, 2R1F

*TPM or CSC indicated as reported in publication, but actual method shown in parenthesis.

Careful experimental design and the use of appropriate controls are important concerns for the use of GJIC assays. Many of these assays are highly sensitive to the density and health of the cells, and the cytotoxicity of the test materials can be an important confounding factor. Another limitation is that nonspecific dye transfer may occur, and it has been suggested that GJIC inhibitors be used to verify that the measurable dye transfer occurred through GJIC, although a specific blocker is not yet available (226, 227). It also should be noted that TPM and CSC produces a fluorescent background and so the cells should be thoroughly washed before flow cytometry analysis; this problem can be minimized by the judicious choice of fluorescent dyes. TPM and CSC have been shown to inhibit GJIC using a variety of different cell models (22, 50, 146, 228, 229). For example, dose-response studies have been conducted in primary hamster tracheal epithelial, and human and rat smooth muscle cells by microinjection-dye transfer techniques (22, 229) and in human bronchial epithelial cells, coronary artery endothelial cells, coronary artery smooth muscle cells, foreskin keratinocytes, WB-344 rat liver epithelial cell lines, and MSU-2 human skin fibroblasts (50, 228).

PREPs have received little attention for GJIC analysis. Using a flow cytometry–based GJIC assay, CSC prepared from low-nicotine or nicotine-free cigarettes under FTC conditions induced greater inhibition than that prepared from the reference cigarettes in normal human bronchial epithelial cells (146). Currently, there are no reports testing the effects of STE on GJIC.

Emerging Technologies for In Vitro *Toxicology.* Recent advances in the "omics" technologies have the ability to yield substantially more information about cellular changes and mechanisms relating to tobacco toxicant exposure. These assays conceptually can provide better data for extrapolation from the *in vitro* cell setting to humans by providing precise information about how a cell does and does not compare to human cells. Included in this category are genome-wide assessments of mRNA expression (transcriptomics), microRNA (miRNA), protein expression (proteomics), epigenetic changes (epigenomics), and small metabolites (metabolomics). Each of these methods produces large data sets, some with hundreds of thousands of data points. Given the vast amount of data that are generated, it is hoped that the comparison of effects in *in vitro* and *in vivo* laboratory studies, and in humans will be possible by elucidating the similarities and differences in these experimental systems. Also, combining technologies leads to a systems biology approach that determines the simultaneous effects on multiple 'omics levels so that particular pathways can be studied from different dimensions. A particular strength of these approaches is the ability to identify or refine pathways for cancer generally, and those affected by tobacco smoke in particular. This could then lead to the discovery of new toxicology assays. Because this is an emerging field, there are few agreed upon methods and methodologic studies for validation. Also the bioinformatic and biostatistical methods widely vary and can provide different results from the same data set. Complicating the assessments further is that the techniques to assess any individual 'omics differ by manufacturer, making laboratory comparisons difficult. Also, the determination of genes, proteins, and metabolic pathways of importance is dependent on somewhat arbitrary criteria, e.g., changes with >2-fold effect and a specific level of statistical significance. However, there could be other analytes with lower fold reductions that might play important regulatory roles, and so a smaller quantitative increase could have a greater biological effect.

Table 14. Summary of Ames test done with cigarette smoke (Cont'd)

Analyte*	Results	Reference
CSC (Cambridge method)	On a revertant/milligram TPM basis there was no statistical difference in the dose-response slopes of the three CSCs in TA98. TA100 produced similar data.	Chen, J 2008 (146)
TPM (Cambridge method), GVP (Solvent trap)	Mutagenic response observed in TA98, TA100, and TA1537. No difference between test and control cigarettes.	Gaworski, CL 2008 (133)
CSC (Cambridge method)	All 10 CSCs were mutagenic in both strains; the low-tar cigarette smoked under the MDPH intense conditions was four times more mutagenic than the same cigarette smoked with the FTC method on a revertant/μg CSC basis	DeMarini, DM 2008 (62)
TPM (Cambridge filters)	Mutagenic activity of the EHC–AMP per milligram TPM was more than 90% lower than that of conventional cigarettes.	Roemer, E 2008 (224)
TPM (Cambridge method)	Differences reported for some strains on a per milligram TPM and per cigarette basis	Patskan, GJ 2008 (127)
CSC (Cambridge method)	Dose-response increase in mutagenicity observed, no difference when reported on a revertant/milligram tar or cigarette between reference and test CSC	Stavanja, MS 2008 (197)
TPM (Cambridge method)	Mutagenic activity of the mainstream smoke condensate from the EHC was lower than Marlboro brands	Werley, MS 2008 (129)
TPM (Cambridge method)	TA98- No statistically significant differences seen between the reference and test cigarettes on either revertant/milligram tar or revertant/cigarette. TA100- cigarettes containing 15% cast sheet showed significantly higher mutagenicity on a revertant/cigarette basis but not a revertant/milligram TPM basis.	Potts, RJ 2009 (84)
TPM (Glass fiber filter) CSC (impaction trap)	TPM smoked according to FTC showed dose-dependent increases, different cigarettes smoked to the same TPM yield showed no significant differences	Roemer, E 2009 (274)

But these genes, proteins, or metabolites could be overlooked. The power of these types of large-scale assessments comes from the identification of effects that have an unknown or unclear biological significance. This also makes the interpretation of data hypothesis generating, rather than hypothesis testing. It is likely, however, that applications and standardization of the various 'omics approaches will improve in the coming years.

mRNA Expression Arrays. Arrays for mRNA expression are widely available for the high throughput analysis of transcripts with the ability to identify specific genes. However, it is difficult to compare array data across laboratories because of the lack of standardization in analysis protocols, assay methods, statistic analyses, and differences among commercially available chips by sensitivity and the number/choice of genes. Given these issues, many journals require authors to include this information in articles with microarray results, via the Minimum Information About a Microarray Experiment checklist, available at MGED Web site.[15]

Changes in gene expression array profiles induced by CSC has been investigated in several studies (42, 48, 230). For example, CSCs prepared from two commercial American cigarette brands under FTC smoking machine conditions were shown to alter the expression of 3,700 genes in cultured normal human bronchial epithelial cells, among 21,329 human genes available on the chip (230). Each condensate changed a unique subset of 1,000 genes, and the authors found that treatment with S9 microsomes resulted in additional changes, including for genes involved in apoptosis, adhesion, and cellular proliferation. In another report, a microarray containing 597 toxicologically relevant human genes, studied in quadruplicate, was used to assess the gene expression changes in cultured human peripheral blood mononuclear cells treated by different concentrations of CSC, TSNAs, benzo(*a*)pyrene, and 4-aminobiphenyl (42). The CSC treatment changed the expression of 260 genes when *t* tests were applied but without a *P* value adjustment for multiple comparisons; this was reduced to 56 genes with a stringent Holm's *P* value adjustment. The CSC changed the expression of many more genes than the other three chemicals, especially for those involved in immune or stress responses, and there were 16 genes differentially expressed by all agents. In another report, using the Affymetrix HG-U133A arrays that assess 22,000 annotated genes, 232 genes were changed in normal human epidermal keratinocytes and three oral cell lines and strains in response to CSC exposure, with 35 genes showing a >2-fold change ($P < 0.005$; ref. 48). These genes were related to xenobiotic metabolism, transport, transcription regulation, and signal transduction. The significantly increased expression of five genes (*CYP1A1, CYP1B1, AKR1C1, AKR1C3*, and *AKR1B10*) also was confirmed by real-time reverse transcription-PCR. No expression microarray studies have been published for STE.

There is growing attention about the role of miRNAs, which are single-stranded RNA molecules controlling gene regulation and carcinogenesis. miRNAs are transcribed, but not translated, and are complementary to mRNA molecules so that protein expression can be downregulated. This recently discovered mechanism is thought to be important for human carcinogenesis and drug discovery. It may also be that that this mechanism is targeted by chemicals, e.g., affected by mutations in DNA affecting transcription of the bases of miRNA. However, the effects of tobacco smoke and ST on miRNA have not been studied.

Proteomics. With the extensive development of protein separation, mass spectrometry, and bioinformatics technologies, proteomics-based techniques are now applicable for conducting toxicology studies for simultaneous changes to a large number of proteins. Proteomics may give a better understanding of an organism's response

to toxic exposures than transcriptomics, because not all transcripts are translated into proteins and proteins can be modified and activated after translation. Approaches for identifying protein markers can be categorized into two principle methodologies: mass spectrometer–based methods and antibody array–based methods. The most commonly used mass spectrometer–based proteomics methods, and their advantages and limitations, were recently reviewed (231-233). TPM prepared from reference 2R4F cigarettes under FTC smoking conditions affected 1,677 unique peptide sequences in the culture supernatants secreted from human microvascular endothelial cells treated using nano-liquid chromatography coupled with high-resolution mass spectrometry (234). Some proteins were significantly differentially expressed, which relate to development, metabolism, communication, and response to stimulus and stress. Other than this study, proteomic approaches have not been used for conducting tobacco toxicology studies, and no proteomic assays have been identified for STE.

Metabolomics. Metabolomics is among the newest 'omic approaches that provides information about the metabolic status of living systems. It therefore became an important component of systems biology, whereby data can be analyzed in the context of genomics, transcriptomics, and proteomics (235-238). Currently available technologies allow for determining the global metabolic profile (a.k.a., the metabolome) by detecting 1,000s of small and large molecules in various media from cell cultures to human biological fluids (235, 238-240). The metabolome consists of metabolic substrates and products, lipids, sugars, small peptides, foreign chemicals (e.g., medicines, toxins, and carcinogens), vitamins and nutrients, and protein cofactors. Therefore, metabolomics provides phenotypic information about the cell's environment and mechanistic pathways that genomics and transcriptomics do not. Metabolomics can be done using various chemical separation techniques (e.g., liquid or gas chromatography or electrochemical array) and then detection and quantitation using nuclear magnetic resonance or mass spectrometry. Metabolomic profiling is inexpensive.

There are generally two strategies for conducting metabolomics studies. One is to conduct a targeted study, where quantitative analysis for specific compounds in various pathways is determined. The other is metabolic profiling, where "fingerprints" of the metabolome are determined to identify new compounds, study multiple pathways, and identify compounds for further study. The potential for metabolomics to study carcinogen and complex human chemical exposures is great (239). Conceptually, numerous tobacco smoke constituents and metabolites can be detected, quantitated, and identified. As a proof of principle using transgenic mice with knockouts for the metabolizing gene CYP1A2, and mice with the humanized CYP1A2 gene, the metabolic profile has been determined for the ubiquitous dietary carcinogen 2-amino-1-methyl-6-phenylimidazo[4,5-b]pyridine (241), revealing 17 urinary metabolites, 8 of which were novel (241). Another example is the detection of the metabolic profiling changes in radiation-exposed TK6 and fibroblast cells, where treated cells had significantly depleted metabolites relating to oxidative stress and DNA repair pathways (242). To date, we are not aware of metabolomic toxicology investigations for cigarette smoker or ST.

Choice of Cell Type and Culture Conditions for Toxicology Studies. The types of cells used for *in vitro* toxicology testing vary greatly from bacterial strains and mammalian immortalized cells to human primary cell cultures. The choice of cell model has many considerations, including the ability to manipulate the cell (e.g., genetically modify them), procurement (e.g., commercial or private availability, and primary strains for humans require surgical tissues or biopsies), replication rates (e.g., faster replicating cells are easier to work with, but the metabolic mechanisms may be very different than in the *in vivo* state), sensitivity and specificity to the test substance, stability of cell lines (a cell line in one laboratory may be phenotypically different from cells with the same name in another laboratory), and culture conditions (loss of function and phenotype change may occur in primary culture; refs. 208, 243). For some assays, for example those that require cells to be arrested in mitosis (22), a rapid proliferation rate is required, and so this is limited to lymphocytes in humans, some mammalian strains, and immortalized cell lines (e.g., CHO cells). Also, the metabolites formed from endogenous enzymes might be different for humans and other cell models, and so these differences might hinder accurate interpretations (28).

Conceptually, the cells that come from humans would be the most informative to screen changes in tobacco products and smoke. It is possible to develop assays from primary cell cultures from blood and target organs, such as normal human bronchial epithelial cells or normal oral keratinocytes (146, 162, 244). Obtaining fresh tissue to develop normal cell primary cultures from human subjects can be challenging for investigators without clinical collaborations, but currently, normal human bronchial epithelial cells, bronchial smooth muscle cells, oral keratinocytes, and hepatocytes are available commercially from several sources. A possible limitation for using primary human cell cultures, or cell lines derived from a single person, is that there is wide interindividual variation among humans for essentially all cellular functions, so that studying a cell strain from only one individual might not yield the same results for cells from other people (245-249). However, this human diversity also is a strength for inferring human responses when strains are tested simultaneously from different individuals. Thus, there should be consideration to using cell strains from several humans so that a range of *in vitro* toxicologic response can be offered. How the sensitivities and specificities of cell lines and human cell strains compare has not been well tested. Recent studies comparing CAs in CHO cells and in cultured human lymphocytes showed that the test substances were more commonly positive in the CHO cells than the human cells (184). This indicates that the former may be overly sensitive to predicting genotoxic effects in humans. Given that immortalized cell lines have various genetic defects that allow for immortalization, this is likely to be the case for other comparisons.

Normal human primary cells have been successfully used for tobacco research, such as for cytotoxicity (23, 48, 50, 105, 144, 146, 155, 162), cell cycle analysis, apoptosis (114, 146) and oxidative stress (250), GJIC (22, 50, 146),

and Comet (146). Some assays, such as GJIC and apoptosis can only be done at very early passage of the primary culture, making widespread applicability more difficult. Cultured lymphocytes are frequently used to assess CA, Comet, and SCE (66, 251, 252).

Immortalized normal human cell lines have been established for the use in toxicology studies, such as the immortalized human oral keratinocytes (253), the bronchial epithelial cell BEAS-2b (90), and the EBV-immortalized lymphoblast (254), but the rapid proliferation rates and other changes can provide very different results that must therefore be interpreted with caution (253, 255). It also is possible to establish *in vitro* toxicology assays from human tumor cell lines, and these lines are often easy to study, but their rapid proliferation rates and other genetic abnormalities also can provide different results from normal human primary cells. Cell lines transfected with cytochrome P450 genes to enhance carcinogen metabolism have been developed and can also be a good model to study the role of P450 metabolism in the toxicity of cigarette smoke (98, 256). This obviates the need for adding S9 metabolic enzymes and provides some level of specificity for a class of toxins within the test substrate. Cells have been transfected with specific cytochrome P450s, such as CYP3A4,CYP2E1, CYP1A1,CYP1A2, and CYP2A6 (257). Although these assays can focus metabolic assessments and implicate specific mechanisms through cytochrome P450s, the relation to the *in vivo* setting is unclear because a number of cytochrome P450s can be induced or inhibited by cigarette smoke (258).

Among the most commonly used cell lines from nonhuman sources are immortalized from experimental animals, especially the CHO cells. The advantages of this cell line are that the protocols for SCE, CA, and neutral red assays are well established and that this cell line has shown sensitivity in detecting cigarette smoke–related toxicity. Other cell lines that are available are the Syrian hamster embryo, Chinese hamster lung, and the mouse lymphoma lines. Recently, a transgenic big blue mouse cell line has become available for genotoxicity testing (259). The disadvantages of immortalized cell lines are that their changes in cell function, metabolism, and genetic makeup may provide different results from normal primary cell cultures or false-positive results. In a report of an recent European Centre for the Validation of Alternative Methods workshop, cell lines such as the CHO cells and mouse lymphoma TK cells were found to have a high false-positive rate (low specificity; refs. 208, 260). The reason for this is that these cells have certain characteristics making them prone to DNA damage, such as altered *p53* status, chromosome instability, and DNA repair deficiencies (208). To reduce false positives in genotoxicity tests, it is recommended to use the cell systems that are *p53* and DNA repair proficient, have defined phase 1 and phase 2 metabolism and a broad set of enzyme forms, and can be used within the appropriately set limits of concentration and cytotoxicity (208).

Effects on Toxicology Studies for TPM/CSC Generated by Different Machine Smoking Conditions. Smoking machine puff profiles can affect toxicology results because they alter how the tobacco burns. Foy and coworkers (61) tested ultralight cigarettes and Eclipse with a variety of toxicology assays and used different smoking machine regimens. Tar yields and toxicity increased with increasing puff volume and frequency, except for the HC method that also has 100% ventilation hole blocking. Ames mutagenicity followed similar patterns. Rickert and coworkers (141, 217) compared cytotoxicity and mutagenicity between CSCs generated by the ISO and HC methods. They reported that although the more intensive HC smoking conditions gave higher mainstream TPM yields on a per cigarette basis, the CSC from three reference cigarettes generated under ISO conditions were more cytotoxic and more mutagenic than those generated under the HC conditions, when the results were reported on a per milligram TPM basis, likely due to the effects of increased ventilation (217). Similarly, Roemer and coworkers (139) reported that cytotoxicity by the neutral red assay and mutagenicity using both mainstream TPM and the GVP were higher for eight commercial cigarettes, three reference cigarettes, and one prototype cigarette smoked under MDPH conditions compared with FTC/ISO conditions, on a per cigarette basis, but the opposite effects were reported on a per milligram TPM basis. In a study using the MLA, TPM generated under FTC and MDPH smoking conditions did not indicate statistically significant differences in the mutagenic activity between the two sets of smoking conditions on a per milligram TPM (205). Thus, although the studies indicate that there are differences among these smoking protocols, it is unclear from the published literature if these differences are due to puff volume, puff frequency, or ventilation. Internal tobacco company documents indicate that all of these have an effect, where in addition to increased ventilation and increasing mutagenicity per milligram of tar as discussed above, changing puff volume and puff frequency affects mutagenicity.[16] Foy and coworkers simultaneously assessed increased puff volume and puff frequency without changing ventilation on cytotoxicity and mutagenicity (61). They reported that there was little difference on a per milligram TPM basis, but was increased on a per cigarette basis for mutagenicity, and no change for cytotoxicity for either reporting method.

Discussion

In vitro toxicology assays have been extensively used over several decades to assess the biological effects of chemical exposures in general, and for cigarette smoke in particular. The effects of STEs have been less thoroughly investigated. The most commonly used assays have differing mode of actions, assessing effects on cytotoxicity, proliferation, cell cycle control, genotoxicity, and epigenetic effects. TPM, CSC, and STE can be used as test substances, often with the addition of metabolizing enzymes. Cell models that have been used range from yeast and bacteria to primary human cell strains. Most smoking machine protocols used for toxicology studies are limited to the FTC regimen, and it is unkown how TPM or CSC can provide different toxicological results. Different extraction methods have not been sufficiently tested for ST. Intuitively, the assessment of tobacco products will need a battery of assays that assess different modes of action with several smoking machine protocols, but specific recommendations for this cannot be made at this time; such a battery will need to

be developed using validated assays for the assessment of tobacco product comparisons.

Extrapolating *In vitro* Toxicologic Studies to Experimental Animals and Humans. *In vitro* toxicologic analyses have been developed for two primary purposes, which are to screen for chemical exposures that might cause disease in humans, and to test hypotheses about mechanisms of disease etiology. For tobacco product design changes, product comparisons have been done, but is almost a unique application. Generally, toxicology assays provide little information about toxic potency of test substances because the results may be particular to the test system. Moreover, they are not designed to compare relative toxic potencies, but rather were developed with high sensitivity and low specificity for detecting harmful signals (149). For example, it is known that *in vitro* tests frequently yield false-positive results compared with *in vivo* animal studies (28, 29, 185). Although in some regulatory contexts, dose-response effects are considered in the context of potency (261), there has only been limited study for tobacco products. DeMarini and coworkers (24) recently assessed the relative potency of CSC from different cigarettes and found that the relative ranking of toxicity by the type of assay was highly variable.

The extrapolation of *in vitro* toxicology data to human risk is complicated. Some of the reasons for this include the use of cells and modes of action where: (*a*) the mode of action and/or metabolic conditions in the cell culture model may not exist in humans; (*b*) chemicals may exert their carcinogenic effects in humans via nongenotoxic mechanisms for which there are very limited toxicology assays; (*c*) many cell models have mutations and increased cell proliferation that are not present in normal human cells; (*d*) many cell models do not have cellular processes that are present in humans (e.g., DNA repair or detoxification pathways); and (*d*) the effects in cell cultures and humans occur at different levels of exposure (27, 29). Another important limitation is that toxicology assays assess short-term exposures and immediate effects, whereas cancer develops in humans over a long exposure and latency period. Omics approach have an appeal because cellular changes can be assessed at lower levels of exposures in human cell strains at early time points without too much perturbation of normal cell function.

To assess the extrapolation potential from the *in vitro* setting to human cancer risk, studies generally consider several steps separately, namely how predictive are *in vitro* tests of *in vivo* animal tumorigenesis studies, and then *in vivo* studies to human risk. *In vitro* models that provide positive results for genotoxic damage best predict tumorigenesis outcomes in experimental animal studies qualitatively, or semiquantitatively (29). But, a negative result in an *in vitro* toxicology study is less reliable (262, 263). For example, mutations found in the Ames test predicted carcinogenicity in laboratory animals with only a 47.5% sensitivity and an 88% specificity, and genotoxic carcinogens were more likely to be rodent carcinogens, but many rodent carcinogens are nongenotoxic (264).

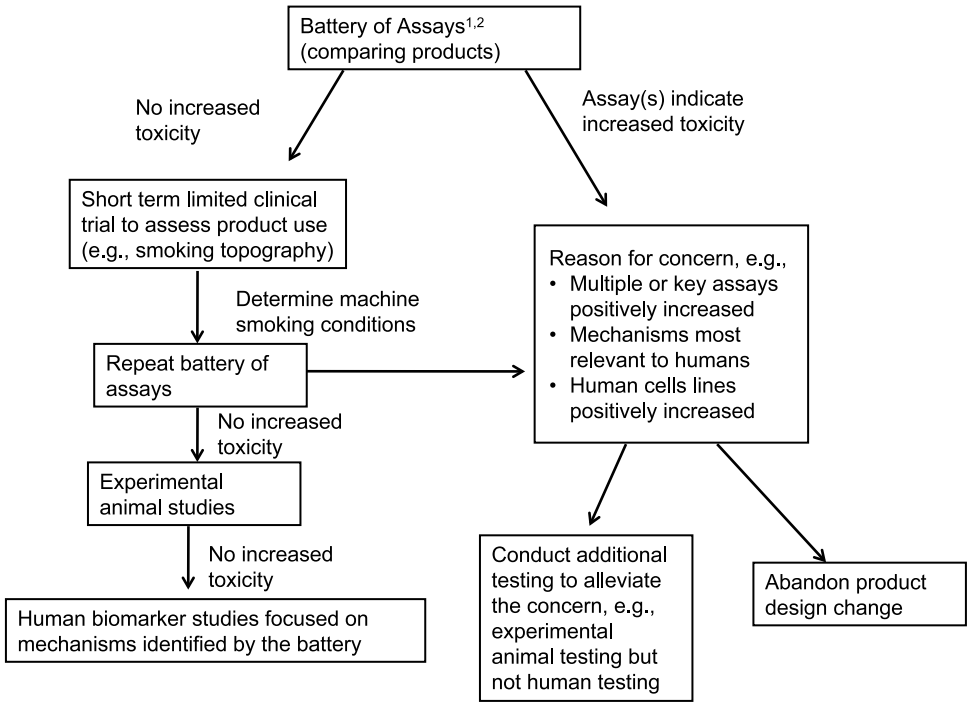

Figure 1. Proposed algorithm for using a toxicologic battery. [1]This algorithm only applies to *in vitro* toxicology testing, and it is assumed that other studies will be done assessing the physical design and chemical analyses. [2]Choice of assays may be informed by smoke chemistry testing.

Table 15. Criteria for validating an assay

Criteria	Explanation
Sensitivity	This is the limit of detection for the assay that is reproducible. Often it includes 95% confidence intervals.
Specificity	This is a measure of how accurate the test is for quantifying a result. For example, if there are interfering substances or multiple contributors to the mode of action result due to test conditions, then the specificity is reduced.
Precision	This is measure of reproducibility. For example, does the assay results similar on different days. Often this is reported as a coefficient of variation.
Accuracy	This is a measure of how close the results are compared with some known true value, assuming that this can be determined.
Ruggedness	This is another measure of reproducibility, but includes as assessment of results by different technicians, machines, columns, etc.
Linearity	This is a measure of the dose responsiveness of the assay. For example, it assesses the performance of an assay over a range of exposures that overlap the expected range of exposure for a particular test condition. Linearity tends to decrease off at the high and low end of exposures.

Some studies suggest that consideration of multiple assays yields greater predictivity (265), but only if these compounds are mutagenic, which of course is the case for many, but not all, tobacco smoke and ST constituents (265-268). Chemicals that do not react with DNA and are nonmutagenic are carcinogenic in experimental animals <5% of the time.

Developing Batteries of Testing and Interpretations for Assessing Tobacco Products. A battery of assays is needed to assess tobacco product changes because no single assay can detect all genotoxic or nongenotoxic compounds (28). If *in vitro* toxicology tests become sufficiently sensitive to report relative potency, then this could be done with a higher level of confidence to assess tobacco product design changes. To do this, there are several considerations that need to be made about the individual assays within a battery, namely (*a*) reproducibility, (*b*) level of cytotoxicity that may confound assay results, (*c*) number of pathways under study and if these represent both genotoxic and nongenotoxic mechanisms, and (*d*) how the results compare to prior assessments (e.g., quantitative levels).

The sensitivity and specificity of batteries for comparing tobacco products is dependent on a number of factors in addition to the choice of particular assays. These relate to how they are interpreted when integrating the data, which can lead to a false negative or positive conclusion, e.g., a compound is considered to be genotoxic or more genotoxic when any result for a single assay within a battery is positive, rather than if a compound is considered genotoxic only when several assays are positive (207). For example, when assessing combinations of 4 genotoxic tests, Kirkland and coworkers (207) showed that for 202 animal carcinogens, a sensitivity value ranged from 46.3% to 92.6%, depending on if one, two, or three assays were positive, but the specificity for the battery to be negative for 96 noncarcinogens was low, especially if the evaluation criteria included only some of the tests being positive. In a recent review about tobacco products by DeMarini et al. (24), it was concluded that the Ames testing and the Comet assay provided sufficient data qualitatively in a combined assessment, where micronucleus, MLA, and CA had no additional value. However, in that article, the assays provided different relative potencies and how this would impact the assessment of a tobacco product design change was not considered. Recommendations for non-tobacco chemical and medication assessments have been made, which might be useful to inform a process for tobacco, although relative potencies are not a consideration. The International Conference on Harmonization of Technical Requirements issued a useful guidance for *in vitro* assessments of medications that can inform tobacco studies (269). The battery recommended by them is (*a*) a test for gene mutation in bacteria, (*b*) an *in vitro* test with cytogenetic evaluation of chromosomal damage with mammalian cells or an *in vitro* mouse lymphoma tk assay, and (*c*) an *in vivo* test for chromosomal damage using rodent hematopoietic cells (the latter *in vivo* mutation assays are not reviewed herein). However, this battery, as described above, is overly sensitive and have reduced specificity. Other recommended batteries require as many as six assays, such as by the European Scientific Committee on Cosmetics and Non-Food Products, although the basis for the recommendation for six assays versus some other number is not clear (207). For tobacco studies, a CORESTA *In vitro* Toxicology Task Force in 2002 provided guidelines to the tobacco industry for product stewardship (62). The recommended tests included the Ames assay, MLA, micronucleus or CA, and theneutral red uptake assay. However, for any of these batteries, an important aspect of developing a battery of *in vitro* tests is to develop standardized methods to apply those assays, which have been recommended in a variety of regulatory contexts (207).

An algorithm would need to be developed for the interpretation of a battery of *in vitro* tests for tobacco product toxicity. The goal of the algorithm would be to identify concerns that would preclude human testing, allow for the choice of experimental animal models based on mechanistic data identified from the batteries, and ultimately inform human testing and the choice of biomarkers based on modes of action (e.g., specific genotoxic mechanisms). The decision process following the testing can occur in one of several ways, as previously described (29, 270). However, there would be a fundamental difference for tobacco products compared with previously developed algorithms, because one tobacco product would be compared with another, and the batteries would be repeated through an iterative process where a product would be tested under arbitrary smoking machine conditions before human testing, and then

again under human smoking conditions. For example, if the smokers' actual use of a product results in very different puff profiles than originally tested, then the battery would be repeated and include the generation of CSC, TPM, WS, and GVP under those conditions. The decision tree would be based on a safety assessment focused on whether the new design is more toxic than the comparator (e.g., the original design, a conventional product, or a PREP). An increase in toxicity could lead to the abandonment of the product design or additional animal testing to provide a basis for continuing the product design change. In the latter case, the experimental animal studies would provide evidence for why the *in vitro* results would not be predictably important for mammalian and human risk. Fig. 1 provides a framework for assessing *in vitro* batteries that indicates increasing levels of concern based on assay results and interpretations, including structure activity relationships and weight of evidence, as suggested by Thybaud and coworkers (29). For tobacco product design changes, the algorithm would include comparisons to other products, and increasing concerns would lead to abandoning the design. It also is assumed that there will be other studies that include physical design analysis and chemical analyses.

Proposed Criteria for Assay Validation. Validation of an assay can mean different things to different people. There are generally two types of validation. The first is for validation of an assay from a laboratory perspective and is quantitative, e.g., sensitivity, specificity, precision, accuracy, ruggedness, and linearity (dose response). The second type of validation refers to relevance, namely how informative is the assay from a biological perspective.

Table 15 indicates various criteria to consider for laboratory validation. To assist with precision and ruggedness, controls that do not vary over time due to storage or handling conditions are needed (e.g., Kentucky reference cigarettes and smokeless tobacco products, chemical standards, and solvent carriers). The type and number of positive and negative controls used for each assay to determine if the assay is still performing in accordance with the above assessments must be determined. Also, there should be quality control and assurance methods for ensuring that the laboratory staff are properly trained and are conducting the assays according to specified protocols (ruggedness). Ultimately, a validated assay from a laboratory perspective should provide similar results for different laboratories. Thus, interlaboratory comparisons also need to be conducted to validate an assay.

The second type of validation refers to the biological relevance of the assay predictivity and the interpretation of the assay. For most contexts and uses of *in vitro* toxicology assays, this is qualitative analysis, but for comparing tobacco products, it would also include a quantitative assessment. This process will consider if the assay accurately represents a mode of action that would be present in humans, if there are confounding variables that affect the results, and how useful are the results for conducting weight-of-evidence decisions. The assessment is mostly qualitative or intuitive because the types of studies needed to show predictivity, e.g., extrapolation of changes in results from *in vitro* cell culture studies to human cancer risk, cannot be scientifically studied.

Research Gaps. Toxicologic analyses of cigarette smoke and ST provide a basis for assessing how tobacco product design changes might adversely impact tobacco users. The available assays assess different modes of action, but each assay presents methodologic challenges and limitations for interpretation. Although a battery of tests that can provide complementary data is needed, which tests will be the most informative and how to interpret the results of a battery are unknown. An important limitation in the available assays is the lack of validation to assess relative potencies of tobacco products. Thus, the application of *in vitro* assays for the assessment of cigarette or ST product design changes, such as for PREPs, is insufficiently developed. There are many research gaps that exist for the evaluation of tobacco product toxicological effects. Although this review has identified numerous research gaps for both cigarette smoke and ST, the major ones include the following:

- The lack of sufficiently validated and sensitive *in vitro* toxicology assays representing a range of modes of action;
- What constitutes the best battery of assays and how to interpret them, including the weighting of assays with different modes of action and reporting of results on a per milligram of tar or ST, per cigarette, or per milligram of nicotine basis;
- What criteria define a validated toxicology assay;
- Whether human cells (primary cultures, genetically engineered cells, or immortalized cells) provide for better sensitivity and specificity for assessing relative potency compared with the commonly used nonhuman and nonmammalian cell assays;
- What are the best ways to mimic human smoking behavior in the laboratory, e.g., use of smoking machine puff profiles for smoking and WS models rather than TPM or CSC;
- Are available assays sufficient to detect changes in unknown toxicity, or are additional assays needed;
- What are the interactions of different chemicals in the complex mixtures of cigarette smoke or STE (e.g., additive, synergistic, and antagonistic effects); and
- Are newer technologies, such as the 'omics assessment, more informative then currently available assays, and can these technologies better inform how an *in vitro* assay might be similar or different compared with human responses.

Perspectives. The tobacco industry regularly makes product design changes to their currently marketed products and are developing PREPs with novel designs. Regulatory authority over tobacco products has recently been given to the Food and Drug Administration, which includes the mandate to evaluate manufacturers' claims, and the authority to mandate performance standards for smoke emissions and exposure. *In vitro* toxicology methods will be needed to evaluate the impact of these in the context of safety evaluations and ensuring that new products are not more toxic than existing products. But there is limited information about how to compare products

with *in vitro* assays and interpret results in the context of both performance standards and health claims. Although the concept of harm reduction for tobacco products is feasible, significant research is needed to develop the laboratory methods to assess changes in specific modes of action and inform decisions about human studies and human cancer risk.

Disclosure of Potential Conflicts of Interest

P.G. Shields serves as an expert witness in tobacco litigation cases on behalf of plaintiffs. The other authors disclosed no potential conflicts of interest.

Acknowledgments

We thank Dr. Harvey Clewell (The Hamner Institutes for Health Sciences, Research Triangle Park, NC) for his advice on this manuscript.

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