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REVIEW

The impact of tobacco additives on cigarette smoke toxicity: a critical appraisal of tobacco industry studies

O impacto dos aditivos do tabaco na toxicidade da fumaça do cigarro: uma avaliação crítica dos estudos patrocinados pela indústria do fumo

El impacto de los aditivos del tabaco en la toxicidad del humo del cigarrillo: una evaluación crítica de los estudios de la industria del tabaco

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Abstract

Cigarette production involves a number of substances and materials other than just tobacco, paper and a filter. Tobacco additives include flavorings, enhancers, humectants, sugars, and ammonium compounds. Although companies maintain that tobacco additives do not enhance smoke toxicity and do not make cigarettes more attractive or addictive, these claims are questioned by independent researchers. This study reviewed the studies on the effects of tobacco additives on smoke chemistry and toxicity. Tobacco additives lead to higher levels of formaldehyde and minor changes in other smoke analytes. Toxicological studies (bacterial mutagenicity and mammalian cytoxicity tests, rat 90 days inhalation studies and bone-marrow cell micronucleus assays) found that tobacco additives did not enhance smoke toxicity. Rodent assays, however, poorly predicted carcinogenicity of tobacco smoke, and were clearly underpowered to disclose small albeit toxicologically relevant differences between test (with tobacco additives) and control (without tobacco additives) cigarettes. This literature review led to the conclusion that the impact of tobacco additives on tobacco smoke harmfulness remains unclear.

Smoking; Tobacco-Derived Products Publicity; Toxicity

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Introduction

Modern cigarettes are extensively engineered and optimized nicotine-delivery systems, and their production involves a number of substances and materials in addition to tobacco, paper and a filter. Components of cigarettes other than tobacco are generally called "ingredients", while the term "additive" is used for substances (with the exception of tobacco and pesticide residues), *"...the intended use of which results or may reasonably be expected to result, directly or indirectly, in its becoming a component or otherwise affecting the characteristic of any tobacco product ..."* (US Food and Drug Administration definition) [1].

Prior to 1970, the tobacco industry used few additives in cigarettes [2,3]. Currently, the industry acknowledges using 600 or so additives in the manufacture of cigarettes [2,3,4,5]. Among the substances that are commonly added to tobacco products are flavorings and enhancers (e.g., cocoa, licorice, menthol, fruit extracts), humectants (e.g., propylene glycol, glycerol, sorbitol), various sugars and ammonium compounds. Collectively these ingredients are referred to as "casings" [4]. Moreover, at a later stage of production, perfume-like volatile substances (e.g., plant essential oils) in an alcohol base, known as "top flavors" or "toppings", are also applied to tobacco mixtures to enhance their flavor and pack aroma [4]. It is reported that "casings" correspond to between 1% and 5% of the weight of cigarette tobacco, and "toppings" to about 0.1% [4].

Tobacco additives are mostly used in American blended cigarettes made with Burley-type tobacco. Virginia-type tobacco cigarettes, which are primarily composed of only one type of tobacco, contain few additives. Burley- and Virginia-type tobaccos undergo different processes of curing [4]. The Burley-type tobacco is allowed to dry at ambient temperature in ventilated barns over a period of 4 to 8 weeks, a long process of curing (air curing) that gives the product a low sugar and high nicotine content. The curing of Virginia tobacco, on the other hand, takes place at higher temperatures in heated barns over shorter periods (5-7 days), a process (flue curing) that quickly inactivates carbohydrate hydrolysis enzymes of tobacco leaves thereby giving this type of tobacco a high sugar and a medium to high nicotine content.4 According to tobacco companies, additives are used to replace sugar lost during Burley tobacco air curing, and to give the blended tobacco product a consistent taste and aroma, and a "sensorial signature". Cigarettes made of Burley (blended) tobacco dominate the market in the United States, Brazil, and other Latin American countries and most of Europe (except for the United Kingdom and a handful of other countries), while Virginia cigarettes are preferred in Canada, the United Kingdom, Australia, Ireland, some Eastern European countries, China, and a few other Asian countries.

Tobacco company documents and reports disclosed by litigation (The Legacy Tobacco Documents Library, LTDL; https://industrydocuments.library.ucsf.edu/tobacco/) strengthened public health scientists' suspicions that such a diversity of tobacco additives is incorporated into cigarettes to make them more attractive, palatable and desirable to potential consumers [2,3,5,6,7,8,9,10,11]. By doing so, tobacco additives would facilitate smoking initiation and maintenance thereby increasing prevalence of smoking and tobacco-related diseases in the population.

Although the industry denies any pharmacological activity of tobacco additives and maintains that they by no means make cigarettes more attractive and addictive, there are a number of indications to the contrary [2,3,5]. Actually, the preponderance of evidence shows that tobacco additives do in fact increase tobacco product appeal and palatability particularly for young people. To make nicotine delivery more acceptable to the smoker, it is necessary to use tobacco additives to attenuate the alkaloid bitterness and harshness. Industry documents indicated that levulinic acid was used to augment nicotine yields while reinforcing perceptions of smoothness and mildness [12]. A recent review and analysis of tobacco industry documents suggested that cigarette manufacturers used pyrazines to increase product appeal, easing smoking initiation and discouraging quitting [13]. Along the same line, menthol, a commonly used tobacco additives, was reported to produce increases in respiratory frequency, a higher respiratory volume and a deeper smoke inhalation. Menthol is a local pain-relieving agent and it is fair to say that it contributes to "smooth" tobacco smoke [6]. By reviewing the literature on sugars as tobacco additives, Talhout et al. [8] concluded that there are consistent indications that sugars mask tobacco smoke harshness and throat impact of tobacco smoke [8,9,10]. Moreover, the authors pointed out that the sweet taste and agreeable smell of caramelized sugars are appreciated in particular by starting adolescent smokers [8]. Nonetheless, Philip Morris International (PMI) researchers [14]

who reviewed "publicly available studies" on the use of sugars as tobacco ingredients concluded that, although causing some differences in smoke composition (e.g. increase in formaldehyde), addition of sugars "*did not lead to relevant changes in the activity in in vitro and in vivo assays*" [14] (p. 244). The industry scientists reiterate tobacco companies' claims that sugars are added to American-blend cigarettes merely to replenish sugar lost during Burley tobacco curing, and that sugar addition by no means increases the inherent risk and harm of smoking [14]. To support the industry's allegations that the addition of sugars does not alter smoking prevalence, Roemer et al. [14] cited a single study by Lee et al. [15] (an ecological-designed cross-sectional study) comparing smoking prevalence between markets with predominantly American-blend (with sugars and tobacco additives) and Virginia-type tobacco (without addition of sugars and few tobacco additives) [15]. Because of the high risk of bias and many possible (uncontrolled) confounding factors, "epidemiology" studies based on cross-country comparisons are not suitable to provide an adequate answer to this question.

The tobacco industry also maintains that tobacco additives do not enhance the inherent toxicity of cigarette smoke. The impact of additives on tobacco smoke toxicity, however, remains unclear [11].

According to the World Health Organization Framework Convention on Tobacco Control (WHO-FCTC) each party shall propose guidelines for testing and measuring the contents and emissions of tobacco products and shall implement effective measures for such testing, measuring and regulation (WHO-FCTC, articles 9 and 10). Since Brazil ratified the WHO-FCTC, the country is committed to implement tobacco control measures [11,16]. As far as tobacco control is concerned, one of the measures that would reduce the prevalence of smoking, and by doing so the occurrence of tobacco-related diseases, is a ban of ingredients and tobacco additives that make tobacco products more attractive and addictive [11,16].

An international expert working group on tobacco additives nominated by the Brazilian Health Regulatory Agency (Anvisa) reviewed the scientific literature, and Brazilian Tobacco Industry Association (ABIFUMO) and agency reports and concluded that the toxicology data available for tobacco additives were insufficient to support companies' claims that they do not enhance tobacco smoke harmfulness [7,17].

Whether or not additives enhance the harmfulness of tobacco smoke is a challenging question for toxicologists. Unless the design of predictive toxicological studies meets some methodological requirements, they are unlikely to provide a satisfactory answer to this question. First, since cigarette smoke is intrinsically quite toxic, demonstrating that additives lead to a significant albeit small increment of (or a small decline of) tobacco smoke toxicity depends on whether toxicity endpoints measured in in vitro or in vivo assays are valid, sensitive and display clear dose-response relationships. Researchers should also be aware that the statistical power to detect a difference depends on the sizes (N) of compared groups. To reveal a small increment over the smoke baseline toxicity the β-error (type-II) estimated for the experiment must be small and thus large control (without additives) and experimental (with additives) groups are required. Second, to assess the contribution of tobacco additives to overall smoke toxicity, toxicologists have to test both the unburned additives and their pyrolysis products. This may prove to be a hard problem because the chemistry of additive pyrolysis remains largely unexplored [5,6]. Third, a comprehensive toxicological evaluation of tobacco additives must include inhalation toxicity tests. Although a small amount of smoke condensate can be eventually swallowed after being deposited on the mucous membranes of the oral cavity and lung bronchial trees (from where bronchial epithelium ciliary movement transports it back to the pharynx), harmful effects of smoking result predominantly from the inhaled tobacco smoke. Fourth, smoking habits result in chronic exposures to smoke toxicants and thus tobacco additives must undergo chronic toxicity testing including long-term rodent carcinogenicity assays. Chronic inhalation toxicity and carcinogenicity assays are methodologically complex, time-consuming, and extremely expensive. Fifth, during cigarette manufacture mixtures of many – rather than a few – tobacco additives (some of which are themselves complex mixtures) are usually employed. Tobacco combustion generates an undetermined number of pyrolysis products from casing and top flavor ingredients that make cigarette smoke an even more complex mixture of constituents. It follows that, according to current estimates, cigarette smoke contains over 4,600 compounds including many proven or suspected carcinogens; i.e., substances classified as "proven" (1), "probably" (2A), and "possibly" (2B) carcinogenic hazards according to the International Agency on Cancer Research (IARC) classification of human

cancer hazards [5,10,18,19,20,21]. Predicting the contribution of single chemicals to the toxicity of multi-component mixtures, and assessing the human health risks posed by exposure to complex mixtures are among the most challenging topics for toxicology research in the 21st Century. Regarding tobacco additives, a question arises as to whether or not additives and their pyrolysis products in the smoke mixture of constituents interact in a way that results in an increase in their overall toxicity compared with the sum of the toxic effects of the individual components of the mixture. To elucidate this question, not only the entire mixture including the tobacco blend plus additives, but also the individual additives themselves must be tested.

Finally, from a public health perspective allocating research funds to evaluate the "safety" of tobacco additives may seem purposeless and ethically questionable. Cigarettes belong to a special class of consumer product that, while they bring no clear benefit to users and the community, pose a substantial risk to active and passive smokers' health. Moreover, tobacco smoke is highly detrimental to health irrespective of whether burned tobacco contains additives or not. It is hard to justify killing a large number of animals merely to demonstrate that tobacco additives enhance, do not change or slightly attenuate the inherent toxicity of smoking.

This literature review addressed the effects of tobacco additives on smoke chemistry and toxicity. The authors critically appraised the strengths and limitations of studies selected for the review. Proposals for tiered testing schemes for the toxicological evaluation of tobacco additives were also given consideration.

Materials and methods

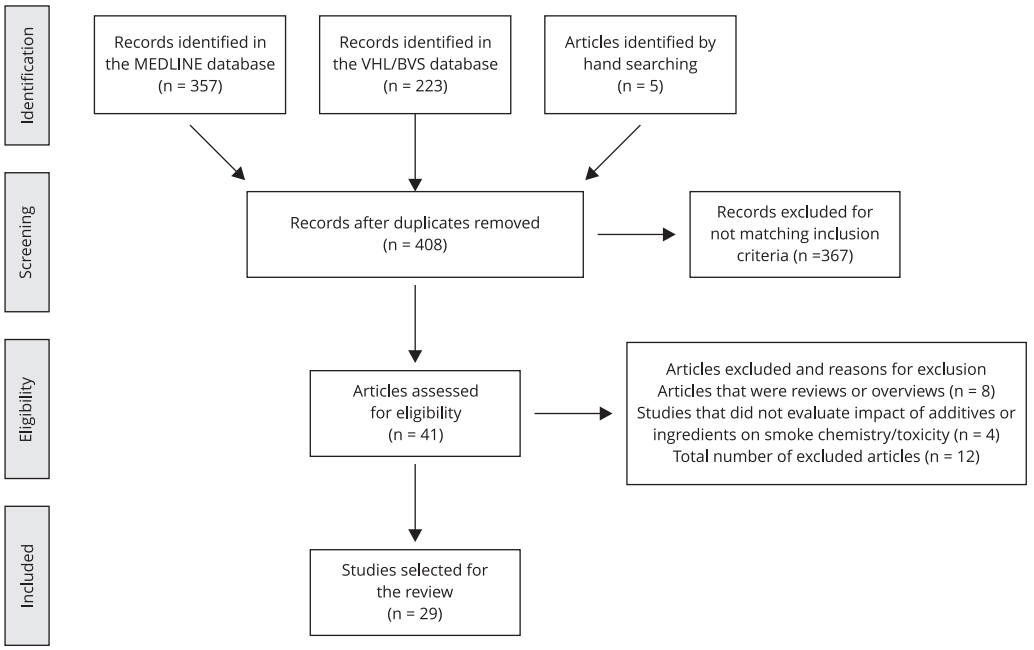
The MEDLINE and Virtual Health Library (BVS) electronic databases were searched using the search string "tobacco additives OR tobacco ingredients". The search covered the period from the inception of the electronic database to 2 August 2015. Reference lists of selected articles, the working group report, and technical documents elaborated by Anvisa and ABIFUMO were also reviewed for potentially eligible studies [17]. There was no restriction regarding the language of the article. An effort was made to retrieve full-text articles of potentially relevant studies. Two researchers separately screened the titles and abstracts for inclusion and exclusion and independently reviewed the articles selected for integral reading and analysis. Articles were excluded according to the following a priori established exclusion criteria: (1) commentaries; (2) reviews or overviews of the literature; (3) theoretical studies; and (4) observational studies on human populations. The a priori eligibility criteria for the studies to be reviewed were as follows: (1) studies that investigated effects of additives on smoke chemistry and/or toxicity; and (2) studies that employed experimental methods. A flow chart of the literature search and selection of articles is shown in Figure 1. A potential limitation of this systematic review is a possible publication bias. Almost all studies on the chemistry and toxicity of mainstream smoke identified in the databases that were searched were sponsored by the tobacco industry (Tables 1 and 2). It is possible that studies that produced results that were unfavorable to the tobacco industry's commercial interests remained unpublished

Results and discussion

Although many tobacco ingredients and additives have been used in the manufacture of cigarettes since the 1970s, studies on the "safety" of additives have predominantly been published in the past two decades (Tables 1 and 2). Overall results of this literature search revealed that the industry's efforts to demonstrate that tobacco additives in current use are "safe" rely primarily on two complementary experimental approaches: (1) evaluations of the effect of single ingredients or mixtures of additives on tobacco smoke chemistry, with a focus placed on the levels of "Hoffman analytes"; and (2) investigations of the impact of additives on the in vitro mutagenicity and cytotoxicity, and in vivo sub-chronic toxicity of cigarette mainstream smoke.

Figure 1

Selection of studies for the review on the impact of tobacco additives on smoke toxicity, PRISMA flow diagram [73].



Effects of tobacco additives on cigarette smoke chemistry

Reviews by Paschke et al. [22] and Rodgman [23,24] examined tobacco industry data generated in the 1950s, 1960s and 1970s. The authors – both prominent researchers from tobacco companies – reviewed studies found not only in medical, toxicological and chemical public databases but also in in-house databases and concluded that flavoring additives, casing materials and humectants produced no significant increases in the cigarette mainstream smoke of either the polycyclic aromatic hydrocarbon (PAH) content, or the benzo[a]pyrene (B[a]P) content [4,23,24]. This review identified a set of more recent studies assessing the effects of ingredients and additives on the levels of a larger group of constituents of toxicological concern (the Hoffmann analytes) in the cigarette mainstream smoke.

The so-called "Hoffmann analytes" comprise 44 or so compounds of toxicological concern found in tobacco mainstream smoke. Of the Hoffmann analytes, only tar, nicotine and CO are produced in mg per cigarette, 29 compounds (formaldehyde, benzene, acetaldehyde, 1,3 butadiene and others) are in the μg/cigarette while the remainders are found in ng/cigarette amounts. The Hoffmann analytes are so termed in recognition of Dietrich Hoffmann's prominent contribution to the field of tobacco carcinogenicity [25]. During his highly productive life, Hoffmann (1924-2011) published a number of analytical studies on the carcinogenic constituents of tobacco smoke [25]. Many scientists from the industry, agencies and academia believe that a significant decrease in the levels of Hoffmann analytes in tobacco smoke could result in less health hazardous cigarettes [4].

As summarized in Table 1, the industry studies invariably suggested that single additives and added mixtures of ingredients have no effect or only a minor influence on the levels of Hoffmann analytes in mainstream smoke. A study by Rustemeier et al. [26], however, showed an enhancement of the yield of total particulate matter (13% to 28%) and increases in the yields (per cigarette) of several smoke constituents in cigarettes containing tobacco additives mixtures. When yields of individual

Table 1

Experimental studies on the effects of tobacco ingredients and additives on smoke chemistry.

Study (sponsor)	Description	Findings/Conclusions
Rustemeier et al. [26] (PMI)	Tested effects of 333 ingredients on the levels of 55 mainstream smoke constituents identified by IARC as worthy of carcinogenic concern.	The total particulate matter yield increased (13 to 28%) for all cigarettes containing tobacco additives. Increase in the amount relative to total particulate matter was noted for HCN, Cd, formaldehyde, resorcinol and Pb.
Baker et al. [27] (BAT)	Effects of 450 tobacco additives on the levels of 44 Hoffmann analytes in mainstream smoke. Control vs test cigarettes, cigarettes were machine-smoked; various analytical methods (GC, GC-MS, HPLC and others); all the analytes measured in the same laboratory at the same time.	Effects of tobacco additives on CO and total particulate matter were non-significant in most cases and not >10% for any tobacco additives mixture.
Baker et al. [28] (BAT)	Effects of 29 casing ingredients and 3 humectants on the levels of "Hoffmann analytes" in mainstream smoke. Control vs test cigarettes, cigarettes were machine-smoked; various analytical methods (GC, GC-MS, HPLC and others); all the analytes measured in the same laboratory at the same time.	Formaldehyde levels increased up to 26mcg (73%) for casing mixtures containing sugar. Added glycerol increased acrolein levels by 26%. Small increases for other Hoffmann analytes.
Stavanja et al. [54] (RJR)	Effect of substitution of honey (5% wet weight) for invert sugar as a casing material in cigarettes on selected mainstream smoke constituent yields.	Substitution of honey (5% wet weigh) for invert sugar had no significant impact on mainstream smoke chemistry.
Carmines & Gaworsky [55] (PMI)	Effect of GLY (3.2%-8.4%/cigarette) on yields of 38 selected analytes in mainstream smoke.	Glycerin increased total particulate matter. On a total particulate matter basis, glycerin (6.2, 8.4%) increased acrolein (9%) and decreased acetaldehyde, propionaldehyde, aromatic amines, nitrogen oxides, tobacco specific nitrosamines, and phenols.
Carmines et al. [56] (PMI)	Effect of licorice extract (1.5%-12%) on yields of selected analytes in mainstream smoke.	On a total particulate matter basis, block licorice extract (12.5%) increased PAH, arsenic, lead, phenol and formaldehyde while licorice extract powder (8% tobacco) increased PAH, phenol and formaldehyde.
Stavanja et al. [57] (RJR)	Effect of HFCS (3%, 4%, 5%) on yields of selected analytes in mainstream smoke.	HFCS produced small increases in formaldehyde, (*p*-+ *m)* – cresol levels and acetone, and small decreases in 4-(methyl-nitrosamine)-1-(3-pyridyl)-1-butanone (NNK) levels. Authors' conclusion was up to 5% HFCS to cigarette does not alter mainstream smoke chemistry.
Lemus et al. [58] (PMI)	Effect of vanillin (0, 67-3109ppm) on yields of 49 analytes including 5 metals in mainstream smoke.	Addition of vanillin to tobacco (up to 3109ppm) did not influence mainstream smoke chemistry.
Gaworsky et al. [59] (PMI)	Effect of PS (0, 0.15%-3.7%) on yields of selected analytes in mainstream smoke.	PS (3.7%) decreased total particulate matter, CO, HCN, 2-nitropropane, and TSN yields, while increasing nicotine, 1,3-butadiene, isoprene, and some PAHs. Authors concluded that high levels of PS alter the burning rate leading to alterations in mainstream smoke chemistry.
Stavanja et al. [60] (RJR)	Effect of $(NH_4)_2PO_4$ (0.5%-1%) and Urea (0.2%-0.41%) on yields of selected analytes in mainstream smoke.	Addition of $(NH_4)_2PO_4$ and urea increased tar HCN, nicotine, hydroquinone ammonia, and catechol, and, on a mg of "tar" basis, increased ammonia and decreased formaldehyde. Authors concluded that addition of tobacco ingredients did not influence mainstream smoke chemistry.
Gaworski et al. [61] (Altria)	Effect of PG (0, 4%, 7%, 10%) on yields of 41 analytes in mainstream smoke.	Addition of PG decreased levels of nicotine and some other analytes in mainstream smoke.
Gaworski et al. [62] (Altria)	Effect of 95 individual tobacco ingredients on yields of selected analytes in mainstream smoke.	High levels of some tobacco ingredients altered the quantity of some analytes in mainstream smoke.
Coggins et al. [63] (Altria)	Effect of 10 aromatic carbonyl compounds (100-10,000ppm) on yields of selected analytes mainstream smoke.	Addition of tested carbonyl compounds did not alter mainstream smoke chemistry.
Coggins et al. [64] (Altria)	Effect of 8 aromatic and aliphatic alcohols (100-24,000ppm) on yields of selected analytes in mainstream smoke.	Addition of eugenol produced several dose-related decreases in the levels of some analytes in mainstream smoke.

(continues)

Table 1 (continued)

Study (sponsor)	Description	Findings/Conclusions
Coggins et al. [65] (Altria)	Effect of 11 carbohydrates and natural product tobacco ingredients on yields of selected analytes in mainstream smoke.	Addition of carbohydrates increased mainstream smoke formaldehyde levels. D-sorbitol and sucrose induced 60%-80% reductions in the levels of some analytes in mainstream smoke while only minimal changes were noted with addition of other tobacco ingredients.
Coggins et al. [66] (Altria)	Effect of 10 Cocoa-derived tobacco ingredients on yields of selected analytes in mainstream smoke.	Addition of cocoa-derived tobacco ingredients produced no consistent changes in mainstream smoke chemistry.
Coggins et al. [67] (Altria)	Effect of 8 aromatic/aliphatic carboxylic acids (100-90,000ppm) on yields of selected analytes in mainstream smoke.	Addition of some tobacco ingredients at high levels resulted in sporadic dose-related changes in the yields of some mainstream smoke constituents.
Coggins et al. [68] (Altria)	Effect of $(NH_4)_2PO_4$ (to 50,000ppm); NH_4OH (to 11,160ppm) +$(NH_4)_2PO_4$ on yields of 40 analytes in mainstream smoke.	Substantial reductions in levels of formaldehyde in mainstream smoke. Sporadic alterations of a few other analytes with no evidence of dose-response relationship.
Coggins et al. [69] (Altria)	Effect of 32 essential oils and resins on yields of selected analytes in mainstream smoke.	Addition of tobacco ingredients produced minimal changes in mainstream smoke chemistry, except for peppermint and spearmint oils that caused reductions up to 40%-60% of some analytes.
Coggins et al. [70] (Altria)	Effect of 15 aliphatic carbonyl compounds on yields of selected analytes in mainstream smoke.	Levels of several analytes in mass spectrometry were decreased by addition of glycerol triacetate (GTA, 100,000ppm). No change was noted with the addition of other mainstream smoke.
Coggins et al. [71] (Altria)	Effect of 3 heterocyclic nitrogen compounds (10-10,000ppm) on yields of selected analytes in mainstream smoke.	DEP caused approximately 10% changes in mainstream smoke chemistry. No change was caused by the addition of either AP or total particulate matter.
Coggins et al. [72] (Altria)	Effect of ethylene vinyl acetate, polyvinyl acetate and starch (adhesives) on yields of selected analytes.	There were some differences in the levels of several analytes in mainstream smoke as a function of the amount of adhesive added.

Altria: in 2003 PMI changed its name to Altria Group Inc.; AP: acetyl pyridine; CO: carbon monoxide; Cd: cadmium; DEP: diethlylpyrazine; DMBA: 7,12-dimethylbenz(a)anthracene; GC: gas chromatography; GC-MS: gas chromatography-mass spectrometry; GTA: glycerol triacetate 2,3-diethylpyrazine; GVP: gas/vapor phase; HCN: hydrogen cyanide; HFCS: high fructose corn syrup; HPLC: high pressure liquid chromatograph; IARC: International Agency for Research on Cancer; Inhl std: rat 90-d inhalation (nose-only) exposure study; JTI: Japan Tobacco Inc; LT: Lorillard Tobacco Co.; MN: micronucleus assay in rodent bone marrow, mouse (SENCAR) back skin two-stage carcinogenicity assay (23 or 30 weeks), promoting agent; NR neutral red uptake assay in mouse embryo Balb/c 3T3 cells; NR-COH: neutral red uptake cytoxicity assay with COH cells; PAH: polycyclic aromatic hydrocarbon; Pb: lead; PBS: phosphate buffered saline solution; PG: propylene glycol; PMI: Philip Morris International; PS: potassium sorbate; RJR: RJ Reynolds Tobacco Co.

analytes were normalized to total particulate matter yields (i.e., analytes were evaluated as the amount on equal total particulate matter basis), a reduction in the majority of analytes was noted. Levels of formaldehyde, HCN, cadmium, lead, and resorcinol, however, remained raised even when they were evaluated as the analyte yield relative to total particulate matter yield. According to Rustemeier et al. [26] comparative assessments were based on the smoke constituent amount relative to total particulate matter yield rather than on absolute amount per cigarette because marketed cigarettes are adjusted to specific tar (total particulate matter) yield segments. Further studies by Baker et al. [27,28] on the impact of ingredients/additives on cigarette smoke composition found increases (up to 73%) of formaldehyde (for addition of casing mixtures containing sugars), acrolein (up to 26%, for addition of glycerol) and minor changes of the remaining Hoffmann analytes. Barring the reported increases in total particulate matter yield per cigarette and in formaldehyde, acrolein, Cd, Pb, HCN, and resorcinol yield relative to total particulate matter yield, industry studies showed that the addition of ingredients to tobacco blends produced no significant increases in mainstream smoke constituents. Nonetheless, the "Hoffmann analytes" represent only a small part of the estimated 4,600+ cigarette smoke constituents [4]. Several suspect carcinogens (e.g., furfural, ethylene oxide, propylene oxide, radioactive elements and radicals) are not listed among the assayed 44 smoke constituents [4].

Table 2

Experimental studies on the effects of tobacco ingredients and additives on mainstream smoke toxicity.

Study (sponsor)	Description		Findings/Conclusions	Reviewers' remarks
	Ingredient	Method		
Suber et al. [73] (RJR)	PG (0, 0.16-2.2mg/L)	Rat 90-d Inhl std. (6h/d, 5d/wk)	Increase in the number of goblet cells or in goblet cell mucin content in the nasal passages at medium- and high-exposures. At the highest concentrations PG caused nasal hemorrhage and ocular discharge possibly as a result of dehydration of the nares and eyes.	Study of the inhalation toxicity of PG. It did not investigate the impact of PG on tobacco mainstream smoke toxicity.
Gaworski et al. [34] (LT)	l-Menthol (5,000ppm)	Rat 90-d Inhl std (1h/d, 5d/wk); mainstream smoke (0, 200, 600, 1,200mg total particulate matter/m^3).	Test and control cigarettes caused similar dose-related toxic effects suggesting that the addition of menthol to tobacco produced no additional toxicity.	Statistical power of experiments is unclear.
Gaworski et al. [35] (LT)	150 tobacco ingredients	26-wk mouse back skin test. mainstream smoke (total particulate matter) tested for promoting activity. N = 30-50.	Total particulate matter was a tumor-promoting agent. Incidence, latency and multiplicity of skin tumors were total particulate matter dose-related. No difference was found between control and test cigarettes. Conclusion: added ingredients did not increase mainstream smoke carcinogenicity.	Total particulate matter from control and test cigarettes were skin tumor promoters. Predictive value of the assay is higher for contact carcinogens than for internal organ carcinogens.
Vanscheeuwijck et al. [32] (PMI)	333 tobacco ingredients (3 groups)	Rat 90-d Inhl std (6h/d, 7d/wk); mainstream smoke (150µg total particulate matter/L) N = 10-14	Mainstream smoke exposure-related findings: hyperplasia and squamous metaplasia of respiratory tract epithelium, atrophy of olfactory epithelium and accumulation of pigmented alveolar macrophages, thymus atrophy (males), thicker laryngeal epithelium. No differences between control and test cigarettes. Conclusion: Added ingredients did not increase mainstream smoke toxicity.	Mainstream smoke of control and test cigarettes were toxic and irritant to the respiratory tract. Statistical power of experiments is unclear.
Roemer et al. [29] (PMI)	333 tobacco ingredients (low/high dose)	SA (total particulate matter); NR, Balb/c 3T3 (total particulate matter, GVP).	Total particulate matter was mutagenic. Total particulate matter and GVP were cytotoxic. No differences between control and test cigarettes. Conclusion: Added ingredients did not increase mainstream smoke genotoxicity and cytotoxicity. It is suggested that (setting a statistical power of 80%) SA tests with TA98 and 100 would detect differences of around 20%. NR test would detect differences of around 30%.	Total particulate matter from control and test cigarettes were mutagenic for TA98, 100, 1537 (but not for TA102 and 1535). Total particulate matter and GVP from control and test cigarettes were cytotoxic. Statistical power of experiments is unclear.
Heck et al. [33] (LT)	Glycerin, PG	Rat 90-d Inhl std (1h/d, 5d/wk); mainstream smoke (350mg total particulate matter/m^3).	Mainstream smoke-related findings: diffuse and focal alveolar pigmented macrophages and chronic interstitial inflammation in the lung, laryngeal epithelial hyperplasia, squamous metaplasia, scab formation, and epithelial hyperplasia in the nose. Inhl std showed no relevant differences between control and test cigarettes Conclusion: added glycerin and PG did not increase mainstream smoke toxicity.	Inhl std showed that mainstream smoke from control and test cigarettes caused inflammation and irritant effects on the respiratory tract. Statistical power of experiments is unclear.

(continues)

Table 2 (continued)

Study (sponsor)	Description		Findings/Conclusions	Reviewers' remarks
	Ingredient	Method		
Stavanja et al. [54] (RJR)	Honey (5% wet weigh)	SA (TA98, 100), SCE-COH (total particulate matter). Inhl std (1h/d, 5d/wk); mainstream smoke (0.06, 0.2, 0.8mg/ wet total particulate matter/L). 30-wk mouse back skin test.	SA (TA98, 100), SCE-COH assays, Inhl std and mouse back skin test found no differences between test and control cigarettes. Conclusion: tobacco cased with honey had comparable toxicological activity to cigarettes containing invert sugar.	Total particulate matter from test and control cigarettes was genotoxic and exhibited tumor promoting activity. Mainstream smoke was toxic and irritant to rat respiratory tract. Statistical power of experiments is unclear
Carmines & Gaworsky [55] (PMI)	Glycerin (3.2%-8.4%/ cigarette)	Rat 90-d Inhl std (6h/d, 7d/ wk); mainstream smoke (150µg total particulate matter/L); N = 10. SA (total particulate matter); NR Balb/c 3T3 (total particulate matter, GVP)	SA and NR tests found no differences between mainstream smoke from test and control cigarettes. In vivo micronucleus assay (90-d inhl std) was negative for control and test cigarettes. Mainstream smoke-related findings: hyperplasia and other changes in nose and olfactory epithelium, macrophage accumulation in the lungs and goblet cell hyperplasia/hypertrophy in nasal epithelium. Conclusion: added glycerin did not increase mainstream smoke toxicity.	Total particulate matter from control and test cigarettes were mutagenic. Total particulate matter and GVP from control and test cigarettes were cytotoxic. In vivo MN assay was unresponsive to mainstream smoke. Statistical power of experiments is unclear.
Carmines et al. [56] (PMI)	Licorice extrs (1.5%-12%)	Rat 90-d Inhl std (6h/d, 7d/ wk); mainstream smoke (150µg total particulate matter/L), N = 10, MN test (Inhl std). SA (total particulate matter); NR Balb/c 3T3 (total particulate matter, GVP).	SA and NR tests found no differences between Total particulate matter from test and control cigarettes. Mainstream smoke from cigarettes with 12.5% block licorice extract caused increased incidence and severity of nose epithelium hyperplasia. Conclusion: at levels equal to and lower than 5% licorice extract did not alter mainstream smoke toxicity.	Total particulate matter from test and control cigarettes were mutagenic. In vivo MN assay was unresponsive to mainstream smoke. Statistical power of experiments is unclear.
Renne et al. [31] (JTI)	165 low-use; 8 high-use flavorings	Rat 90-d Inhl std (1h/d, 5d/wk); SA (total particulate matter).	SA found no differences between total particulate matter from test and control cigarettes. Mainstream smoke-related findings: concentration-related hyperplasia, squamous metaplasia, and inflammatory responses in the respiratory tract (decreased or disappeared after recovery period). Conclusion: added tobacco ingredients did not increase mainstream smoke toxicity.	Total particulate matter from test and control cigarettes was mutagenic. Inhl std found total particulate matter concentration-related detrimental effects on respiratory tract epithelial tissue. Statistical power of experiments is unclear.
Stavanja et al. [57] (RJR)	HFCS (3%, 4%, 5%)	SA (TA98, 100), SCE-COH. NR-COH (total particulate matter). Inhl std (1h/d, 5d/wk); mainstream smoke (0.08, 0.26, 0.8mg/ wet total particulate matter/ L). 30-wk mouse back skin test.	SA (TA98, 100), SCE-COH, NR-COH assays, Inhl std and mouse back skin test found no differences between test and control cigarettes. Conclusion: added tobacco ingredients did not increase mainstream smoke toxicity	Total particulate matter from test and control cigarettes was genotoxic, cytotoxic and exhibited tumor promoting activity. Mainstream smoke was toxic and irritant to rat respiratory tract. Statistical power of experiments is unclear

(continues)

Table 2 (continued)

Study (sponsor)	Description		Findings/Conclusions	Reviewers' remarks
	Ingredient	Method		
Lemus et al. [58] (PMI)	Vanillin (0, 67-3,109ppm).	SA (total particulate matter). NR Balb/c 3T3 (total particulate matter, GVP). Rat 90-d Inhl std (6h/d); mainstream smoke (150mg total particulate matter/m^3).	NR and SA assays found no differences between cigarettes with different levels of vanillin. Inhl stds showed no difference between groups. Most of the changes after 90-d exposure were resolved in a 42-day post-inhalation period. Conclusion: vanillin up to 3,109ppm did not alter a broad range of toxicological endpoints.	Statistical power of experiments is unclear.
Gaworsky et al. [59] (PMI)	PS (0, 0.15%-3.7%)	Rat 90-d Inhl std (6h/d); mainstream smoke (150µg total particulate matter/L), N = 10, MN test (Inhl std). SA (total particulate matter); NR Balb/c 3T3 (total particulate matter, GVP).	SA and NR assays found no differences between mainstream smoke from control and test cigarettes. Mainstream smoke-related histopathology findings consistent with those seen in previous Inhl stds. Inhl std showed no relevant differences between control and test cigarettes. Conclusion: Added PS did not increase mainstream smoke toxicity.	Total particulate matter from test and control cigarettes were mutagenic. Total particulate matter and GVP from control and test cigarettes were cytotoxic. Inhl std showed concentration-related detrimental effects of mainstream smoke on respiratory tract epithelial tissue. Statistical power is unclear.
Stavanja et al. [60] (RJR)	$(NH_4)_2PO_4$ (0.5%-1%) Urea (0.2%-0.41%)	SA (TA98, 100), SCE-COH (total particulate matter). Inhl std (1h/d, 5d/wk); mainstream smoke (0.06, 0.2, 0.8mg/wet total particulate matter/L). 30-wk mouse back skin test.	SA (TA98, 100), SCE-COH assays, Inhl std and mouse back skin test found no differences between test and control cigarettes. Conclusion: Added tobacco ingredients did not increase mainstream smoke toxicity.	Total particulate matter from test and control cigarettes was genotoxic and exhibit tumor promoting activity. Mainstream smoke was toxic and irritant to rat respiratory tract. Statistical power is unclear
Gaworski et al. [61] (Altria)	PG (0, 4%, 7%, 10%)	SA (total particulate matter). NR Balb/c 3T3 (total particulate matter, GVP). Rat 90-d Inhl std (6h/d, 7d/wk); mainstream smoke (150mg total particulate matter/m^3).	NR and SA tests found no differences between control and test cigarettes. Inhl stds showed minimal changes caused by added PG most of which were resolved in the 42-day post-inhalation period.	Statistical power of experiments is unclear.
Gaworski et al. [62] (Altria)	95 tobacco ingredients	SA (total particulate matter). NR Balb/c 3T3 (total particulate matter, GVP). 31 tobacco ingredients: Rat 90-d inhl std (1h/d).	SA and NR assays found no differences between control and test cigarettes. Mainstream smoke-related histopathology findings consistent with those seen in previous stds. Inhl std showed no relevant differences between control and test cigarettes. Conclusion: added ingredients did not increase mainstream smoke toxicity	Statistical power of experiments is unclear.

(continues)

Table 2 (continued)

Study (sponsor)	Description		Findings/Conclusions	Reviewers' remarks
	Ingredient	Method		
Coggins et al. [63] (Altria)	10 aromatic carbonyl compounds (100-10,000ppm)	SA (total particulate matter). NR Balb/c 3T3 (total particulate matter, GVP). Ethyl vanillin: Rat 90-d inhl std (1h/d).	SA and NR assays found no differences between control and test cigarettes. Mainstream smoke-related histopathology findings consistent with those seen in previous stds. Inhl std showed no relevant differences between control and test (ethyl vanillin) cigarettes. Conclusion: added ingredients did not increase mainstream smoke toxicity.	Statistical power of experiments is unclear.
Coggins et al. [65] (Altria)	8 aromatic and aliphatic alcohols (100-24,000 ppm)	SA (total particulate matter). NR Balb/c 3T3 (total particulate matter, GVP). Benzyl alcohol, propyl paraben, rum flavor; Rat 90-d Inhl std (1h/d).	Eugenol caused dose-related decrease in GVP cytotoxicity. In all other cases, SA and NR tests found no differences between test and control cigarettes. Inhl std found a few sporadic differences between control and test cigarettes. Conclusion: Added ingds did not increase mainstream smoke toxicity.	Statistical power of experiments is unclear.
Coggins et al. [65] (Altria)	11 Carbohydrates and natural product tobacco ingredients	SA (total particulate matter). NR Balb/c 3T3 (total particulate matter, GVP). 10 ingredients: Rat 90-d Inhl std (1h/d).	Maltodextrin decreased cytotoxicity and plum juice increased it. In all other cases, SA and NR tests found no differences between test and control cigarettes. Inhl stds showed a few sporadic differences between control and test cigarettes. Conclusion: added tobacco ingredients did not increase mainstream smoke toxicity.	Statistical power of experiments is unclear.
Coggins et al. [66] (Altria)	10 Cocoa-derived tobacco ingredients	SA (total particulate matter). NR Balb/c 3T3 (total particulate matter, GVP) 5 cocoa-derived tobacco ingredients: Rat 90-d Inhl std (1h/d).	SA and NR tests found no differences between test and control cigarettes. One of the cocoa tobacco ingredients caused increases in histopathology severity scores (not dose-related). In all other cases, Inhl stds showed no differences between control and test cigarettes. Conclusion: added tobacco ingredients did not increase mainstream smoke toxicity.	Statistical power of experiments is unclear.
Coggins et al. [67] (Altria)	8 aromatic/ aliphatic carboxylic acids (100-90,000ppm)	SA (total particulate matter). NR Balb/c 3T3 (total particulate matter, GVP). Rat 90-d Inhl (1h/d).	Lactic acid caused decrease in cytoxicity. In all other cases, SA and NR tests found no differences between test and control cigarettes. Lactic acid produced dose-dependent reductions in histopathology restricted to the nasal passage. Inhl stds showed no other difference between test and control cigarettes. Conclusion: added ingds did not increase mainstream smoke toxicity.	Statistical power of experiments is unclear.
Coggins et al. [68] (Altria)	$(NH_4)_2PO_4$ (to 50,000ppm); NH_4OH (to 11,160ppm) +$(NH_4)_2PO_4$	SA (total particulate matter). NR Balb/c 3T3 (total particulate matter, GVP). Rat 90-d, Inhl std (1h/d).	Differences when present occurred sporadically with no evidence of dose-effect relationship. Conclusion: added diammonium phosphate and ammonium hydroxide, even at high inclusion levels, have minimal toxicological sequelae.	Statistical power of experiments is unclear.

(continues)

Table 2 (continued)

Study (sponsor)	Description		Findings/Conclusions	Reviewers' remarks
	Ingredient	Method		
Coggins et al. [69] (Altria)	32 essential oils and resins	SA (total particulate matter). NR Balb/c 3T3 (total particulate matter, GVP). 7 tobacco ingredients: Rat 90-d Inhl std (1h/d).	Except for a dose-related reduction in cytotoxicity, a reduction in body weight gain and atrophy of olfactory epithelia for spearmint oil, differences when present occurred sporadically with no evidence of dose-effect relationship. Conclusion: tested essential oils and resins show minimal toxicological sequelae, even at high inclusion levels.	Statistical power of experiments is unclear.
Coggins et al. [70] (Altria)	15 Aliphatic carbonyl compounds	SA (total particulate matter). NR Balb/c 3T3 (total particulate matter, GVP). GTA: Rat 90-d inhl std (1h/d).	GTA (100,000ppm) reduced cytotoxicity and mutagenicity (TA1537 w/S9). In all other cases, SA and NR tests found no differences between test and control cigarettes. Inhl std showed no differences between control and test (GTA) cigarettes. Conclusion: added tobacco ingredients did not increase mainstream smoke toxicity.	Statistical power of experiments is unclear.
Coggins et al. [70] (Altria)	heterocyclic nitrogen compounds (10-10,000ppm)	SA (total particulate matter), NR Balb/c 3T3 (total particulate matter, GVP).	SA and NR tests found no differences between test and control cigarettes.	Statistical power of experiments is unclear.
Coggins et al. [71] (Altria)	ethylene vinyl acetate, polyvinyl acetate, starch (adhesives)	SA (total particulate matter). NR Balb/c 3T3 (total particulate matter, GVP). Rat 90-d Inhl std (1h/d).	NR and SA tests found no differences between control and test cigarettes. Inhl std found no differences between test and control cigarettes. Conclusion: added tobacco ingredients did not increase mainstream smoke toxicity.	Statistical power of experiments is unclear.

Altria: In 2003 PMI changed its name to Altria Group Inc; COH: Chinese hamster ovary cells; GTA: glycerol triacetate; GVP: gas/vapor phase; HFCS: high fructose corn syrup; Inhl std: rat 90-d inhalation (nose-only) exposure study; JTI: Japan Tobacco Inc; LT: Lorillard Tobacco Co.; MN micronucleus assay in rodent bone marrow. Mouse (SENCAR) back skin two-stage carcinogenicity assay (23 or 30 wks), promoting agent: TPA: 12-*O*-tetradecanoyl-phorbol-acetate, initiating agent: DMBA: *7,12*-dimethylbenz(*a*)anthracene; NR: neutral red uptake assay in mouse embryo Balb/c 3T3 cells; PBS: Phosphate buffered saline solution; PG: propylene glycol; PMI: Philip Morris International; PS: potassium sorbate; RJR: RJ Reynolds Tobacco Co; SA. *Salmonella* microsome assay with tester strains TA98, 100, 102, 1535, 1537; with and without addition of rat liver S9 (Aroclor 1254-induced).
NR-COH; neutral red uptake cytoxicity assay with COH cells; SCE-COH: sister chromatid exchange assay in Chinese hamster ovary cells.

Effects of tobacco additives on cigarette smoke toxicity

Table 2 shows that the impact of tobacco additives on cigarette mainstream smoke toxicity was investigated through *in vitro* mutagenicity (Ames test) and mammalian cytotoxicity (Neutral red uptake) assays [29,30,31], and *in vivo* sub-chronic (90 day) inhalation toxicity studies with rats [31,32,33,34]. In addition to these investigations (Table 2), a sub-chronic toxicity (26-week) study tested smoke condensates from cigarettes with and without tobacco additives for tumor promoting activity in the two-stage carcinogenicity test on mouse back skin [35].

In vivo toxicity tests

In sub-chronic inhalation toxicity tests, rats were nose-only exposed to cigarette smoke generated by smoking-machines adjusted to deliver smoke with a target and fairly constant total particulate matter concentration to both test (cigarettes with additives) and control group animals (cigarettes

without tobacco additives). Similarly, in all *in vitro* assays, and in the *in vivo* mouse back skin-painting assay, tested doses expressed the amount of smoke condensate (total particulate matter). It follows that *in vitro* and *in vivo* toxicity tests compared test and control cigarette smokes on the basis of equal total particulate matter amounts. Under these experimental conditions, a tobacco additive-produced increase in total particulate matter yield per cigarette [26] did not exert any influence on test results. According to tobacco industry toxicologists, adjustment of smoke total particulate matter-yield in the test would be the most realistic approach to the question addressed by these studies because commercial cigarettes are adjusted to specific total particulate matter (or "tar") market segments.

A common limitation of all experiments conducted to investigate the effects of additives on tobacco smoke toxicity is the unclear statistical power to detect differences between control and test cigarette smokes when a difference truly exists. Roemer et al. [29] suggested that *Salmonella* TA98 and TA100 mutagenicity tests and NR uptake cytoxicity assays were capable of detecting differences of around 20% and 30%, respectively. Owing to the small group sizes and the marked variability of toxicity endpoint measures, it is fair to think that *in vivo* sub-chronic toxicity studies listed in Table 2 were also underpowered to detect small albeit toxicologically relevant differences between the test and control groups.

The duration of sub-chronic (90-d) inhalation toxicity tests (Table 2) is obviously insufficient to disclose long-term carcinogenic effects of tobacco mainstream smoke. Along this line, two-year toxicity and carcinogenicity studies with rodents remain the primary experimental method by which chemicals are identified as having the potential to cause cancer in humans. Long-term rodent toxicity assays are important to unveil cancer hazards, whenever exposures to chemicals reaching the systemic circulation occur regularly over a substantial part of an individual's life. Epidemiological evidence suggesting that smoking increases the risk of cancer in multiple organs such as lung, larynx, oesophagus, mouth, pharynx, bladder, pancreas, kidney, liver, stomach, bowel, cervix, ovary, nose, and some types of leukemia [18,36,37] adds to the importance of systematically examining a large number of tissues after long-term rodent exposures. Nonetheless, chronic carcinogenicity studies of inhaled mainstream smoke with rats, mice, hamsters, dogs and non-human primates have produced negative or only marginally positive results for cancers of the lungs and other tissues [38,39,40,41,42]. The negative results for tobacco smoke in chronic inhalation toxicity/carcinogenicity assays are at odds with the abundant epidemiological evidence proving that active (and passive) smoking considerably increases risks of lung cancer (and also of tumors of other organs) in humans. This discrepancy between findings from observational epidemiology studies in humans [18,36,37] and data from chronic carcinogenicity inhalation studies with a diversity of laboratory animal species [38,39,40,41,42] has remained largely unexplained. A mouse back skin-painting test confirmed the tumor-promoting effect of tobacco smoke condensates (tar) [43]. As far as tobacco smoke is concerned, however, dermal contact is not a toxicologically relevant route of exposure, and skin tumors (including benign papillomas) may not be representative of other organ malignancies.

Rat sub-chronic inhalation studies did not detect differences between smoke from test (with tobacco additives) and control (without tobacco additives) cigarettes, but they did reveal noncancerous toxic effects related to smoke exposure such as chronic interstitial inflammation in the lungs, and hyperplasia, squamous metaplasia and other pathologic alterations of the epithelium which were particularly severe in the upper part of the respiratory tract (Table 2). Rodent 90-day inhalation toxicity studies, therefore, have mainly found non-cancerous lesions of the respiratory tract caused by tobacco smoke.

As obligate nasal-breathers, rodents inhale tobacco smoke exclusively though their noses irrespective of the inhalation method (inhalation chamber or nose-only exposure) used in the study. Continuous and heavy exposure of nasal cavity to tobacco smoke explains the severe irritant and toxic effects on the upper respiratory tract and nasal epithelium. Contrasting to rodents, adult humans have the ability to breathe through either the nasal or the oral cavity, and active smokers inhale tobacco mainstream smoke primarily by the oral cavities, while passive smokers breathe side-stream smoke mainly through the nasal cavity. The severe irritant effects on the nasal epithelium of rats after sub-chronic inhalation exposures are unlikely to occur in active smokers. Long-term inhalation toxicity studies with rodents, on the other hand, have failed to reveal the known lung cancer hazards posed by tobacco smoke.

In vivo genotoxicity tests (mouse bone marrow micronucleus assays) have also been used for testing tobacco additives. A major problem with using this *in vivo* gentoxicity (clastogenicity/aneugenicity) test for revealing a possible change in tobacco smoke toxicity is its poor response or even unresponsiveness to tobacco smoke [44]. Industry toxicologists justify the inclusion of such known smoke-unresponsive tests in a test battery for tobacco additives arguing that it seeks to ensure that this "*lack of micronucleus activity was maintained with the addition of the ingredient*" [45] (p. 122).

***In vitro* toxicity tests**

The contribution of tobacco additives to overall smoke toxicity has been investigated with bacterial mutagenicity tests (*Salmonella*/microsome assay) and cytotoxicity assays (e.g. neutral red uptake). The guide for testing toxicity of tobacco ingredients from the Deutsches Institut für Normung (DIN, German Institute for Standardization) recommends an *in vitro* assay for chromosomal damage in the testing battery (e.g. mammalian cell micronucleus test) [4,46]. Nonetheless, comparisons of toxicities of smoke condensates from test cigarettes (with additives) with those from control cigarettes (without tobacco additives) seldom included the latter genotoxicity assay.

The predictive value of *in vitro* toxicity assays used to compare the smoke yielded by test (with tobacco additives) and control (without tobacco additives) cigarettes is limited by the poor metabolic competence of bacterial and cell test systems, and also by the difficulty in mimicking the *in vivo* exposure conditions. Roemer et al. [29], for instance, performed a mammalian cell (mouse embryo BALB/c 3T3 cells) cytoxicity (Neutral red uptake) assay and calculated EC50 concentrations in the absence of extrinsic metabolic activation. BALB/c 3T3 cells, however, have a poor metabolic competence and do not reproduce the biotransformation undertaken by smoke constituents after *in vivo* exposures. The *Salmonella* assays, on the other hand, were generally conducted both in the presence and in the absence of extrinsic metabolic activation systems (i.e. Aroclor 1254-induced rat liver post-mitochondrial fraction) [29,31]. In the mammalian cytoxicity assay, both the total particulate matter and the water-soluble portion of the gas/vapor phase (GVP) trapped in PBS were administered to cells (EC50s for GVP were comparable to those obtained for total particulate matter) [29]. *Salmonella* mutagenicity assays, however, tested total particulate matter but not GVP yielded by test and control cigarettes [29,31].

Testing strategies to assess tobacco additive toxicity

Although stakeholders have agreed that tobacco additives require a toxicological assessment, there has been no consensus among agencies, the industry and independent scientists on the extent of toxicity testing of tobacco additives. As commented in the introduction to this review, on account of theoretical and practical constraints the toxicological assessment of tobacco additives remains a challenging question.

Based on current scientific knowledge, it is plausible to think that data from *in vitro* and *in vivo* toxicity assays are of limited value to predict smoking-related risks and harm to human health, including lung cancer. Consequently, comparative toxicity testing approaches may not be sufficient to make inferences on the contribution of specific additives and/or mixtures of additives to the overall toxicity of tobacco smoke. This conclusion was reached by experts of the Committee on Carcinogenicity of Chemicals in Food, Consumer Products and the Environment (UK-COC) who re-affirmed that current toxicological studies are not suitable to predict the impact of tobacco additives on smoke toxicity [47]. According to the UK-COC: "*…the studies available that assessed the contribution of individual or mixed ingredients or additives to the overall toxicity of tobacco products are inadequate to assess the risks posed by conventional cigarettes, so it is not possible to assess the modulation of that risk resulting from inclusion of additives. The relationship between effect (an increase in biomarker) and exposure is also poorly understood*" [47]. As aforementioned, Anvisa's expert working group on tobacco additives also concluded that available scientific evidence (as to August 2014) was insufficient to support any conclusion that additives have no impact on tobacco smoke harmfulness [7,17].

Tobacco industry (PMI) researchers advanced a tiered approach to assess the "safety" of cigarette ingredients/additives [45]. Industry testing scheme tiers are maximum use levels (concentration rela-

tive to cut filler weight, ppm) as follows: tier 0 (up to 0.025ppm): only literature review and QSAR (Quantitative Structure-Activity Relationships); tier 1 (15ppm), tier 0 plus pyrolysis and/or analysis of volatile organic compounds; tier 2 (90ppm): tier 1 plus smoke chemistry (18 smoke constituents); tier 3 (300ppm), tier 2 plus a broader smoke chemistry (Hoffman analytes); tier 4 (3,000ppm), tier 3 plus *in vitro* mutagenicity assays (*Salmonella* assay; NR uptake cytotoxicity; mouse lymphoma assay); and tier 5 (> 3,000ppm), tier 4 plus 90-day inhalation toxicity study (rats) and *in vivo* bone marrow micronucleus assay (mice) [45].

The industry approach involves establishing cut-off points based on anticipated amounts of the ingredient added to tobacco products (as the "level of concern") to trigger an additional amount of toxicity testing. This tiered testing scheme was inspired on the Threshold of Toxicological Concern (TTC) concept, a pragmatic approach developed for assessing the risk of compounds of known chemical structure for which no compound-specific toxicity data are available [48,49,50,51,52]. The TTC concept assumes that human exposure to chemicals below the corresponding TTC are very unlikely to cause any adverse effect. According to Dempsey et al. [45], the additive level triggering *in vivo* inhalation studies (30,000ppm) (tier 5) was *"derived from ingredient inhalation studies sponsored by PMI over more than a decade"* [45] (p. 126). That is, 95% of PMI-conducted inhalation studies did not show adverse effects of tested ingredients up to concentrations as high as 30,000ppm. The cut-off points of intermediate tiers (15, 90, 300, and 3,000ppm) were derived from defined percentiles along cumulative distribution curve of the NOAELs, while the cut-off for the lowest tier 0 (0.025ppm) was based on TTC levels previously established for genotoxic substances [45].

Standing primarily on the ingredient amount added to a tobacco product, the industry's tiered testing approach dramatically reduces the number of current tobacco additives potentially requiring further *in vitro* and *in vivo* testing. Moreover, even at the highest level of concern (tier 5) only two *in vivo* tests would be required for tobacco additives, an inhalation (90-day) toxicity "with emphasis on irritant changes in the respiratory tract" and a mouse bone marrow micronucleus assay.

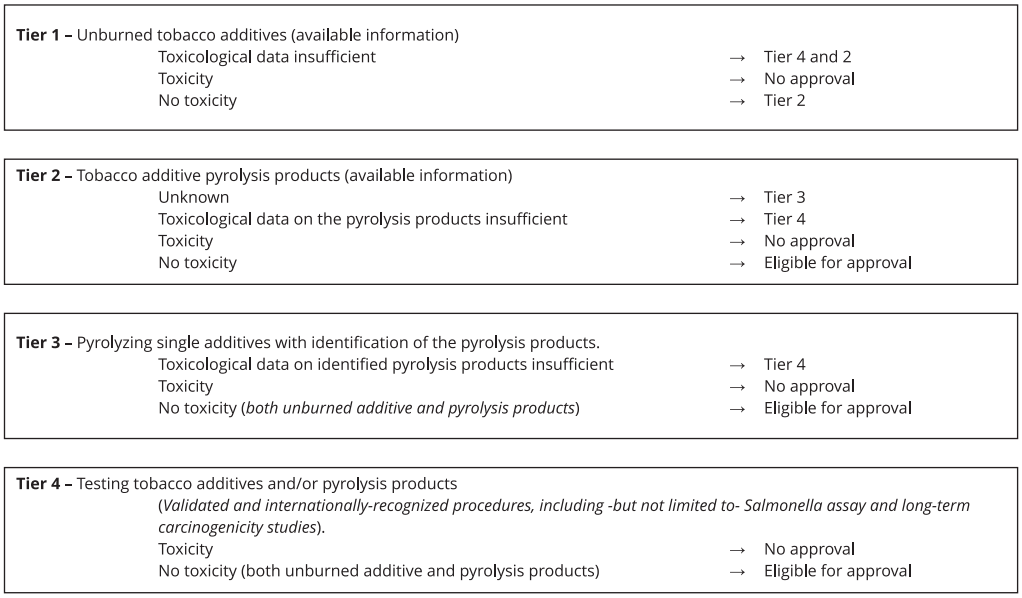
Since adequate toxicological data including chronic inhalation toxicity, genotoxic potential and long-term carcinogenicity tests do not exist for most tobacco ingredients and their pyrolysis products, tobacco companies' testing schemes would allow for the incorporation of small amounts of a great number of untested substances in the manufacture of tobacco products. The industry proposal is based on the implicit assumption that of dozens or even hundreds of low-level untested tobacco ingredients and their pyrolysis products, each and every substance does not interact with each other in additive or synergistic ways.

The German Cancer Research Center (DKFZ: Deutsches Krebsforschungszentrum) has proposed a less conservative tiered approach for testing the toxicity of tobacco additives (Figure 2) [6]. In contrast to the industry scheme, the DKFZ approach is based on the principle that, in this particular case, the level of proof of safety must be set very high because tobacco products containing additives bring no health or other benefits to smokers or the general population [6]. The decision-making process associated with the DKFZ tiered testing scheme precludes the incorporation of an ingredient to tobacco if results at any of the tiers point to a detrimental impact of the ingredient addition on overall smoke toxicity. The DKFZ tiered testing scheme is as follows. Tier 1 involves assessing the toxicity of individual additives in their unburned form. If the available toxicological information is insufficient, unburned additives should undergo testing for toxicity (tier 4). Tier 2 of the DKFZ approach involves a toxicological evaluation of pyrolysis products. Again, if available information is insufficient, pyrolysis products should be tested for toxicity (tier 4). Tier 3 involves pyrolyzing the single additive (pyrolysis products are currently unknown) under realistic and standardized conditions. If toxicological information on pyrolysis products identified by the most sensitive analytical method proves to be insufficient, they should undergo testing for toxicity (tier 4). Tier 4 involves testing the toxicity of additives and their pyrolysis products through validated and internationally-recognized procedures such as those described by OECD (Organisation for Economic Co-operation and Development) guidelines (e.g. 471: bacterial mutation test, and 451, long-term carcinogenicity testing) [6].

The DKFZ tiered testing approach was criticized by tobacco-industry researchers. Ruth Dempsey and colleagues argue that the DKFZ tiered-testing scheme would lead to *"a ban of nearly all ingredients, as the combustion of organic materials always leads to some toxicants like formaldehyde"* [45] (p. 125). According to them, this approach could result in banning some ingredients, *"even when they produce far less*

Figure 2

The tiered testing approach proposed by the Deutsches Krebsforschungszentrum (DKFZ, German Cancer Research Center) for evaluating the toxicity of tobacco ingredients and additives [6].



toxicants than tobacco and thus dilute the toxicants in the smoke" [45] (p. 125). Although in theory a (small) dilution effect might eventually occur, in practice no study has proved that single additives or mixtures of additives in current use dilute tobacco smoke toxicants, and/or make cigarette smoke less hazardous to smokers' health.

Since tobacco smoke is a highly complex mixture of toxicants, an upwards (or downwards) adjustment of this background toxicity by low levels of added ingredients may prove difficult to detect in standard toxicity assays. Rat sub-chronic inhalation assays, for instance, failed to detect any effect of tobacco additives on the incidence and severity of respiratory tract histopathology findings (Table 2), although it was demonstrated that smoke levels of formaldehyde (a recognized rodent carcinogen and irritant compound), and a few other toxicants were increased by tobacco additives, particularly by tobacco additives mixtures containing sugars (Table 1) [53].

The testing approach proposed by DKFZ seeks to exclude the incorporation of any foreseeable hazardous substance to tobacco products besides tobacco itself. Taking into account the methodological constraints and challenges, and the uncertainty about the contribution of individual tobacco additives to overall smoke toxicity, the stringent DKFZ testing scheme seeks to err on the side of safety as far as possible.

Concluding remarks

In conclusion, owing to insufficient toxicity testing, and poor predictive value, low statistical power and other methodological constraints of conducted comparative toxicity studies, it remains unclear whether or not single additives and/or mixtures of constituents currently added to tobacco products impact on the overall toxicity of cigarette smoke. Tobacco-related human health risks, however, depend on both the overall smoke toxicity and exposure to tobacco smoke. Regardless of whether

tobacco additives increase or do not alter smoke toxicity, the preponderance of evidence indicates that they make smoking initiation and maintenance easier, thereby contributing to a higher prevalence of smoking and tobacco-related illnesses in the population. A possible enhancing effect of tobacco additives on smoking prevalence, and thus on the prevalence of tobacco-related illnesses, was highlighted in UK-COC experts' report: "*Furthermore, it is possible that additives might alter smoker behaviour, such as to increase product use; this increased exposure would be likely to result in an increased risk*" [47].

Finally, the burden of proof lies with the tobacco companies who have the responsibility to provide scientifically sound evidence supporting any conclusion that additives do not add to overall smoke toxicity and do not enhance the attractiveness, palatability and/or addictiveness of tobacco products. At any rate, based on the best evidence available, it is plausible to think that a ban on the use of tobacco additives in the manufacture of cigarettes would result in progressive declines in the prevalence of smoking and tobacco-related morbidity and mortality. A prompt enforcement of the Anvisa imposed ban on most tobacco additives [11] – pending a final decision by Brazil's Supreme Court – would certainly be a big step towards achieving the public health goal of a first generation of tobacco-free Brazilians in the coming decades.

Contributors

F. J. R. Paumgartten and A. C. A. X. Oliveira participated in the selection and review of the studies, write-up and review of the final article. M. R. Gomes-Carneiro participated in the review of the articles, write-up and review of the final article.

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Resumo

A produção de cigarros envolve uma série de substâncias e materiais além do próprio tabaco, do papel e do filtro. Os aditivos do tabaco incluem conservantes, flavorizantes, intensificadores, umectantes, açúcares e compostos de amônio. Embora as empresas produtoras de tabaco aleguem que os aditivos não aumentam a toxicidade da fumaça e não tornam os cigarros mais atraentes ou viciantes, tais alegações são contestadas por pesquisadores independentes. Os autores realizaram uma revisão dos estudos sobre os efeitos dos aditivos sobre a composição química e toxicidade da fumaça. Os aditivos elevam os níveis de formaldeído e causam pequenas alterações nos níveis de outros analitos medidos na fumaça. Estudos toxicológicos (testes de mutagenicidade e de citotoxicidade em células de mamíferos, estudos da exposição por 90 dias por via inalatória em ratos e ensaios do micronúcleo em células da medula óssea) indicaram que os aditivos do tabaco não aumentam a toxicidade da fumaça. Entretanto, é conhecido que os estudos em roedores falham em predizer o potencial carcinogênico da fumaça do cigarro, e os testes realizados tiveram poder estatístico insuficiente para detectar diferenças pequenas, porém relevantes do ponto de vista toxicológico, entre cigarros experimentais (com aditivos) e controles (sem aditivos). Em conclusão, esta revisão da literatura mostrou que o impacto dos aditivos na toxicidade da fumaça do tabaco ainda permanece por ser esclarecido.

Hábito de Fumar; Publicidade de Produtos Derivados do Tabaco; Toxicidade

Resumen

La producción de cigarrillos involucra un número de sustancias y materiales diferentes al tabaco en sí, papel y filtro. Los aditivos del tabaco incluyen aromas artificiales, potenciadores del sabor, humectantes, azúcares, y compuestos de amonio. A pesar de que las compañías sostienen que los aditivos del tabaco no aumentan la toxicidad del humo y no hacen los cigarrillos más atractivos y adictivos, estas afirmaciones son cuestionadas por investigadores independientes. Este trabajo ha revisado los estudios sobre los efectos de los aditivos del tabaco en la química del humo y su toxicidad. Los aditivos del tabaco conllevan niveles más altos de formaldehído y otros cambios menores en los análisis realizados del humo. Estudios toxicológicos (tests de mutagenicidad en bacterias y citotoxicidad en mamíferos, ensayos de inhalación en ratas 90 días y células del micronúcleo de la médula ósea) mostraron que los aditivos del tabaco no aumentaron la toxicidad del humo. Los ensayos de roedores, sin embargo, no predijeron adecuadamente la carcinogenicidad del humo del tabaco, y no eran claramente suficientes para dar a conocer, sin embargo, las pequeñas, pero toxicológicamente relevantes, diferencias entre el test (con/aditivos del tabaco) y control (sin/aditivos del tabaco) en cigarrillos. Esta revisión de la literatura nos lleva a la conclusión de que el impacto dañino de los aditivos del tabaco en el humo continúa estando poco claro.

Hábito de Fumar; Publicidad de Productos Derivados del Tabaco; Toxicidad

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