

6.1.3 *In vitro* toxicology studies

In vitro toxicology testing is an established and integral component of FDA regulatory oversight across historically-regulated product sectors, providing qualitative and quantitative information on potential adverse effects of products. *In vitro* test methods have evolved to the point that a very high degree of control over experimental conditions is possible. The *in vitro* testing of cigarette smoke and tobacco extracts provides data that is informative and directly relevant to advancement of the current understanding of the mechanisms of tobacco-related diseases (USDHHS 2010; CORESTA IVTF 2004). Data from such studies complement and inform the findings of other product evaluations, *e.g.*, *in vivo* animal and human clinical investigations (LSRO 2007a).

Whereas *in vitro* testing has evolved to a state of maturity that is approaching its direct application in human risk quantitation (White and Johnson 2016), it is at present generally appreciated to have limitations in terms of providing direct, quantitative predictions of human disease risk. It does, however, provide both qualitative and quantitative data that have utility in the development or confirmation of hypotheses that support and explain collateral lines of evidence derived from human clinical and epidemiology studies (Andreoli *et al.* 2003; Allen 2006).

FDA has stated that *in vitro* toxicology testing can provide useful information to address the known and potential toxicities of tobacco products, and thus has utility in evaluation of the range of toxicities of a potential MRTP as compared to other tobacco products on the market (FDA MRTPA Draft Guidance 2012, p. 24). A review published several years ago discussed the strengths and weaknesses of available *in vitro* assays in the comparative assessment of tobacco products, and presents an encyclopedic listing of published papers on both cigarettes and smokeless tobacco products (Johnson *et al.* 2009). In its report to FDA on “Scientific Standards for Studies on Modified Risk Tobacco Products,” the Institute of Medicine (IOM) discussed the utility, as well as the limitations, of *in vitro* toxicity and genetic toxicology tests of potential risk-modified tobacco products in bacterial and mammalian systems. That IOM report provided a brief summary of such work for both smokeless tobacco and cigarettes (see IOM 2012, Tables 3-2 and 3-3). More recently, additional peer-reviewed, published work has accumulated that has further demonstrated the value and utility of contemporary *in vitro* methodologies to advance understanding of disease processes, as well as to accomplish comparisons of the toxicity inherent to different tobacco products (Manuppello and Sullivan 2015).

The primary *in vitro* tests that have historically been used to characterize and compare tobacco products are those that evaluate genotoxicity and cytotoxicity (reviewed in Andreoli *et al.* 2003). As discussed briefly below, the past and current focus on these two manifestations of toxicity is entirely appropriate in light of the current mechanistic understanding of major diseases caused by smoking.

6.1.3.1 Rationale for the use of *in vitro* genotoxicity and cytotoxicity endpoints in comparative evaluations of different tobacco products

The potential mechanisms by which cigarette smoking causes cancer have been intensely studied over the last five decades. While much progress has been achieved in elucidating the mechanism(s) by which smoking causes cancer, the scientific challenge is far from complete. One such mechanism entails the capacity of cigarette smoke or its constituents to damage the structure of an affected cell's DNA in a way that alters the expression of key genes involved in cell division, growth, differentiation and proliferation. The genetic toxicity (genotoxicity) of cigarette smoke, whether caused directly by smoke chemicals or indirectly through reactive species derived from endogenous inflammatory processes, serves as a primary basis for *in vitro* toxicology testing and research in areas relating to tobacco and smoking (DeMarini 2004). Since genetic changes are widely acknowledged to be a major underlying mechanism of cancer initiation, genotoxicity testing of tobacco products has been applied to advance insight into disease mechanisms (Andreoli *et al.* 2003; USDHHS 2010), as well as to make comparisons among different tobacco products (IOM 2012; Manuppello and Sullivan 2015). Genotoxicity assays using cigarette smoke total particulate material (TPM; regarded herein as synonymous with cigarette smoke condensates, CSC) and whole smoke typically produce robust dose-response curves that enable potency comparisons among test articles in a given assay platform. A substantial extant body of published literature representing over 40 years of work on tobacco-related genotoxicity is available to provide a context for comparisons among products.

Test systems that measure structural changes to the genetic material, such as the mammalian cell micronucleus and sister-chromatid exchange assays, and those that evaluate the induction of mutations in target genes, such as the Ames *Salmonella* mutagenesis assay, have proven to be particularly reliable in providing evidence for the clastogenic and mutagenic properties of cigarette smoke.

Cytotoxicity tests assess the capacity of chemicals or mixtures to kill mammalian cells, a process which is believed to play a role in cancer, cardiovascular diseases, and respiratory diseases such as COPD (Rock and Kono 2008; USDHHS 2010; Yeager *et al.* 2016). Chronic inflammatory processes that underlie these diseases, and that occur subsequent to cytotoxic insult *in vivo*, produce not only reactive genotoxic species, but also tissue repair through cell proliferation. Such cell proliferation is known to promote the development of tumors in epithelial tissues such as those lining the human respiratory tract. Tumor promotion has, in fact, been described as the primary etiologic mechanism of smoking-related lung carcinogenesis based on analyses of population data derived from several large cohorts (Hazleton *et al.* 2005).

Thus, a battery of genotoxicity and cytotoxicity assessments can provide data having relevance to disease processes that occur among tobacco-using human populations. A list of well-established *in vitro* assays applied in support of this Application is provided below in Table 6.1.3-1, and includes those that have been most commonly employed in tobacco product evaluations to date. These methods are extensively reviewed by DeMarini 2004 and Johnson *et al.* 2009. A more recent review further documents additional examples of investigations using

these and other emerging *in vitro* test systems in comparative assessments among tobacco products (Manuppello and Sullivan 2015).

Whereas hundreds of *in vitro* studies have been conducted on cigarette smoke and smokeless tobacco in recent decades (Johnson *et al.* 2009), only a relative handful of published papers to date have reported concurrent testing of both types of tobacco products under matched testing conditions. The available evidence to date, however, consistently demonstrates that the relatively low genotoxicity and cytotoxicity of smokeless tobacco products contrasts so starkly with the higher toxicity of combusted tobacco products that minor differences in study protocols beyond those necessary to generate appropriate smokeless extracts or smoke preparations seem unlikely to substantially affect the qualitative findings for the respective product categories.

Table 6.1.3-1: *In vitro* Assays Used to Evaluate Various Tobacco Products

Assay Name	Assay Endpoint	Description
Ames mutagenicity assay / bacterial reverse-mutation	Genotoxicity	The Ames test uses different genetically-engineered strains of <i>Salmonella</i> bacteria to determine whether a particular treatment (with a test chemical or mixture) produces mutations in those bacteria.
Mammalian cell Sister chromatid exchange (SCE) assay	Genotoxicity	The SCE assay detects symmetrical exchanges of genetic material between adjacent chromatids. SCE is a sensitive assay that can detect direct- and indirect-acting genotoxicants.
Mammalian cell Micronucleus (MN) assay	Genotoxicity	The micronucleus assay is used to detect genotoxic damage caused by chemical or physical agents and is capable of detecting damage by both clastogenic and aneugenic compounds.
Mammalian cell Neutral red uptake (NRU) assay	Cytotoxicity	The neutral red uptake assay examines cellular membrane integrity and cellular energy status, as neutral red dye is taken up into lysosomes by living (healthy) cells by an energy-dependent process.

Assay Name	Assay Endpoint	Description
Human Urinary mutagenicity (discussed elsewhere; see Section 6.1.2 of this Application)	Genotoxicity and information on internal exposures to genotoxins from actual product use	A hybrid <i>in vitro</i> + clinical approach explicitly endorsed by the IOM as “an effective and reliable method of quantifying human exposure to mutagens created by combusted tobacco” (IOM 2012, p. 114).

6.1.3.2 Published *in vitro* toxicology studies (mutagenicity, genotoxicity and cytotoxicity) of cigarette smoke and cigarette smoke extracts

A variety of assays utilizing cultured cells have shown that exposure to cigarette smoke or smoke condensate consistently results in adverse effects, as measured using genotoxicity, cytotoxicity, or other endpoints (comprehensively reviewed by [Johnson et al. 2009](#)). Analogous genotoxic and cytotoxic events are similarly well documented in an extensive body of published work from both *in vivo* animal, human clinical and population studies ([USDHHS 2010](#)). The findings of most genotoxicity assays of cigarette smoke TPM¹ or whole smoke are typically robust and dose-related in appropriately-selected test systems having a provision to mimic key metabolic activation processes that are important to the manifestation of toxicity in intact animals or humans. Among the most informative tests of this type that have provided evidence for the genotoxic properties of cigarette smoke are those for mammalian cell micronuclei and sister-chromatid exchanges, and for the multiple types of gene mutations that are efficiently detected in the Ames *Salmonella* assay ([Andreoli et al. 2003](#)). Not only is cigarette smoke condensate genotoxic in nearly all *in vitro* assay platforms in which it has been tested, analogous smoking-associated genotoxic effects have been found in many of the target organ sites for cancer caused by smoking that have been examined to date; including oral/nasal, esophagus, pharynx/larynx, lung, pancreas, myeloid organs, bladder/ureter, and uterine cervix (reviewed by [DeMarini 2004](#)).

A variety of cytotoxicity assays, including the neutral red uptake assay, has also demonstrated a dose-response using cigarette smoke TPM or whole smoke (reviewed in [Johnson et al. 2009](#)). A number of recent reviews have considered the extensive body of literature regarding the *in vitro* toxicology of cigarette smoke ([IARC 2004](#); [USDHHS 2004](#); [Johnson et al. 2009](#); [USDHHS 2010](#)). All of these publications document the fact that cigarette smoke is both cytotoxic and

¹ The term “cigarette smoke condensate” (CSC) is formally and most properly used in reference to machine-generated cigarette smoke particulate material collected by cold trap condensation, whereas smoke particulate material collected on Cambridge glass fiber filter pads connected to a laboratory smoking machine is most properly referred to as smoke “total particulate material” (TPM). In the context of *in vitro* toxicological studies, these terms of reference have frequently been used interchangeably in the published literature. The cigarette smoke test solutions used in the RJRT studies presented in this section were all DMSO solutions of filter-collected TPM. The terms “CSC” and “TPM” may be considered to be synonymous in this section of the Application, as well as in the context of the cited published literature and referenced technical reports.

genotoxic, and promotes other effects consistent with the known harms associated with smoking.

The variety of subjective, social and behavioral factors that affect risks to individual users of cigarettes or smokeless tobacco products are less important considerations for *in vitro* studies that characterize the fundamental toxicologic properties of smokeless tobacco and cigarettes in cross-category comparative assessments. *In vitro* evaluations of this type are certainly most informative when conducted in concert with studies of analytical chemistry, exposure biomarkers and manner of use by consumers in clinical or real-world settings, as has been done in support of this Application.

A number of newer genotoxicity and cytotoxicity assays have been described and applied on an investigational basis in the assessment of smokeless tobacco and cigarette smoke particulate matter (Johnson *et al.* 2009; Manuppello and Sullivan 2015), as well as the whole cigarette smoke aerosol (Thorne *et al.* 2015b). Such assays hold promise for future comparative analyses of tobacco products as they mature to a standardized and validated state of development. The CORESTA Sub-Group on *In vitro* Toxicology Testing (CORESTA 2007) is currently considering the pursuit of proficiency trials for emerging *in vitro* whole smoke aerosol exposure methods. This collaborative, international scientific group has previously overseen the conduct of inter-laboratory evaluations and proficiency trials for a recommended *in vitro* test battery for use in the evaluation of cigarette smoke condensates, including the Ames *Salmonella* mutagenicity assay, the mammalian cell micronucleus assay and the mammalian cell neutral red cytotoxicity assay. These three *in vitro* methods have also been specified by Health Canada as Official Methods T-501, T-502 and T-503 (Health Canada 2004). All of these standardized and well-validated test methods for cigarette smoke testing were performed, along with others, in support of this Application; with modifications appropriate for testing of smokeless tobacco extracts.

6.1.3.3 Published *in vitro* toxicology studies (mutagenicity, genotoxicity and cytotoxicity) of smokeless tobacco

Published *in vitro* toxicology studies of smokeless tobacco to date are fewer in number than studies of cigarette smoke and smoke extracts, and have employed a variety of extraction and testing methods. A particular challenge to investigators has been the standardization of methods across a spectrum of loose and pouched products of differing composition and moisture content. Comparisons among products and tests have been made by expressing results on a “per mg extract” basis, “per mg nicotine” basis, on a “dry-weight tobacco” basis, or on a “product unit as-is” basis. No single approach to making the most informative comparisons among products is appropriate for all purposes, and each may be viewed as offering different insights (Rickert *et al.* 2009).² Summaries of significant published *in vitro* studies of smokeless tobacco are provided in the following text.

² RJRT has not attempted to determine which of the various techniques for testing and data analysis are most appropriate for the evaluation of smokeless tobacco products. Therefore, multiple approaches to both testing and analysis were pursued and are reported herein. RJRT acknowledges FDA’s previously-stated preference for cross-

An early genotoxicity study evaluated both aqueous and methylene chloride extracts of Swedish snus in a battery of *in vitro* assays (Jansson *et al.* 1991). Positive mutagenic responses were reported for methylene chloride extracts in Ames *Salmonella* strains TA98 and TA100 with S9 activation (+S9); and a borderline positive response was indicated in TA1537 +S9. Whereas positive responses were determined as positive linear slope values for the dose-response curves, a visual examination of the data presented as the authors' Figure 1 indicated that the peak revertant colony counts represented less than a doubling of spontaneous revertant counts in all strains and activation conditions. Aqueous extracts were found inactive in all tester strains and S9 conditions. Both the aqueous and methylene chloride snus extracts were found positive in producing sister chromatid exchanges (SCE) in human lymphocytes and chromosome aberrations in V79 hamster cells in the presence of S9 activation. The modest induction of chromosome aberrations by the aqueous extract in the absence of S9 was attributed to the osmotic effects of the high salt content of the tested snus sample, as a follow-up test with a salt-free sample found it to be inactive. Neither the aqueous nor the methylene chloride extract induced point mutations at the HPRT locus in V79 cells or bone marrow micronuclei in orally-dosed mice, and the methylene chloride extract was similarly negative in producing sex-linked recessive lethal mutations in *Drosophila*. None of the assays showed robust positive responses such as those typically seen in similar tests of cigarette smoke. The authors stated that, based upon consideration of their findings in these *in vitro* assays and the accompanying *in vivo* tests with the Carcinogenicity Prediction by Battery Selection mathematical model, that "...the carcinogenic potential of Swedish snus should be considered to be low," and noted that their findings aligned with the low oral cancer rates in Sweden relative to other countries. Since the chemical composition of Swedish snus has changed substantially over the course of the last two decades (*e.g.* significant reduction in TSNA; Österdahl *et al.* 2004; IARC 2007b; SCENIHR 2008; WHO 2008; Stanfill *et al.* 2011), these findings reported 24 years ago by Jansson and colleagues may overestimate the genotoxic potential of contemporary snus products.

A series of related studies on the effects of smokeless tobacco extracts on several cell types, including macrophages, hepatic cells, human oral epithelial cells, and normal human oral keratinocytes have been reported (Bagchi *et al.* 1995; Bagchi *et al.* 1996; Bagchi *et al.* 1997; Bagchi *et al.* 1999). These investigations documented increased production of reactive oxygen species, with accompanying elevations in indices of oxidative stress, lipid peroxidation, DNA fragmentation and apoptosis in cell cultures treated with up to 300 ug/mL of aqueous extracts (1 gram/5 mL) prepared from a standardized chewing tobacco obtained from the University of Kentucky Tobacco and Health Research Institute. Co-treatments with antioxidants, including vitamins C, E and a grape seed proanthocyanidin extract, were found to inhibit the increases in oxidative stress markers induced by smokeless tobacco extracts.

The *in vitro* study of Merne *et al.* 2004 revealed that snus extract did not stimulate cellular proliferation, but did disrupt epithelial differentiation. Treatment of an immortalized epithelial skin cell line (HaCaT cells), for more than 12 days produced morphological changes, which

category comparisons of HPHC levels among tobacco products on the basis of units of consumer use (*e.g.* per cigarette, per pouch smokeless tobacco) and has included such comparisons when practical.

included loss of basal cell layer, apoptotic cells, and impaired cellular adhesion. There were disturbances in the differentiation process; however, there was no increase in cellular proliferation.

Recent published work with snus has reported it to have dramatically lower mutagenic, cytotoxic, and genotoxic activity than has been reported routinely for cigarette smoke TPM. Laytragoon-Lewin *et al.* (2011) conducted experiments that directly compared toxicity of snus to cigarette smoke extract in low-passage normal human endothelial cells and fibroblasts. Cells exposed to cigarette smoke extract exhibited dramatically altered gene expression, strongly inhibited DNA synthesis, cellular abnormalities, and massive cell death. In contrast, snus extract induced only cytoplasmic abnormalities with no significant increase in cell death (Laytragoon-Lewin *et al.* 2011). Furthermore, some of the cytoplasmic abnormalities (such as prominent cytoplasmic vacuoles) observed in the snus-treated cells were similar to those seen in cells treated only with nicotine.

Rickert *et al.* 2009 conducted comparative studies of the chemistry and toxicology of commercial smokeless tobacco products available in Canada, including imported Swedish snus and a number of US-manufactured smokeless products. When the Ames mutagenicity assay, the micronucleus assay for clastogenicity, and the neutral red cytotoxicity assay were applied to smokeless tobacco products, potencies were only a small fraction (less than 10%) of those observed for extracts of mainstream cigarette smoke condensate. Rickert *et al.* 2009 noted that their results showing low mutagenic activity of smokeless extracts are consistent with the finding by Curvall and colleagues that the mutagen levels in snuff users' urine were no higher than those found in the urine of nonusers and abstinent snuff users (Curvall *et al.* 1987).

Coggins *et al.* 2012 reported testing of aqueous extracts of three commercial brands of Swedish snus, an experimental snus, and 2S3 reference moist snuff at varying concentrations in four different assays (Ames, NRU, MN, and the L5178Y *tk*⁺/₋mouse lymphoma cell (MLY) mutagenesis test). Results for snus in the mutagenicity and clastogenicity (Ames, MLY, MN) assays were broadly negative, with occasional positive responses noted, usually at very high and often cytotoxic doses well in excess of those advised in regulatory guidelines. The 2S3 reference moist snuff was unequivocally positive at the highest concentration only in one of the three conditions of the micronucleus assay. Accompanying chemical analyses reported substantially higher levels of NNK, NNN, cadmium and B[a]P in the 2S3 reference moist snuff than in any of the tested Swedish snus products. The generally negative data for snus are contrasted with data historically reported for smoke condensates from combusted cigarette tobacco, in which strongly positive mutagenicity and cytotoxicity has been consistently observed. These findings of minimal, inconsistent genotoxicity for Swedish snus in a laboratory setting concur with the large body of epidemiologic data from Sweden showing that Swedish snus is associated with considerably lower carcinogenic potential when compared with cigarettes.

Misra *et al.* 2014 reported a comparison of the cytotoxic, mutagenic, and genotoxic effects of the total particulate matter from tobacco cigarettes to that of contemporary smokeless tobacco

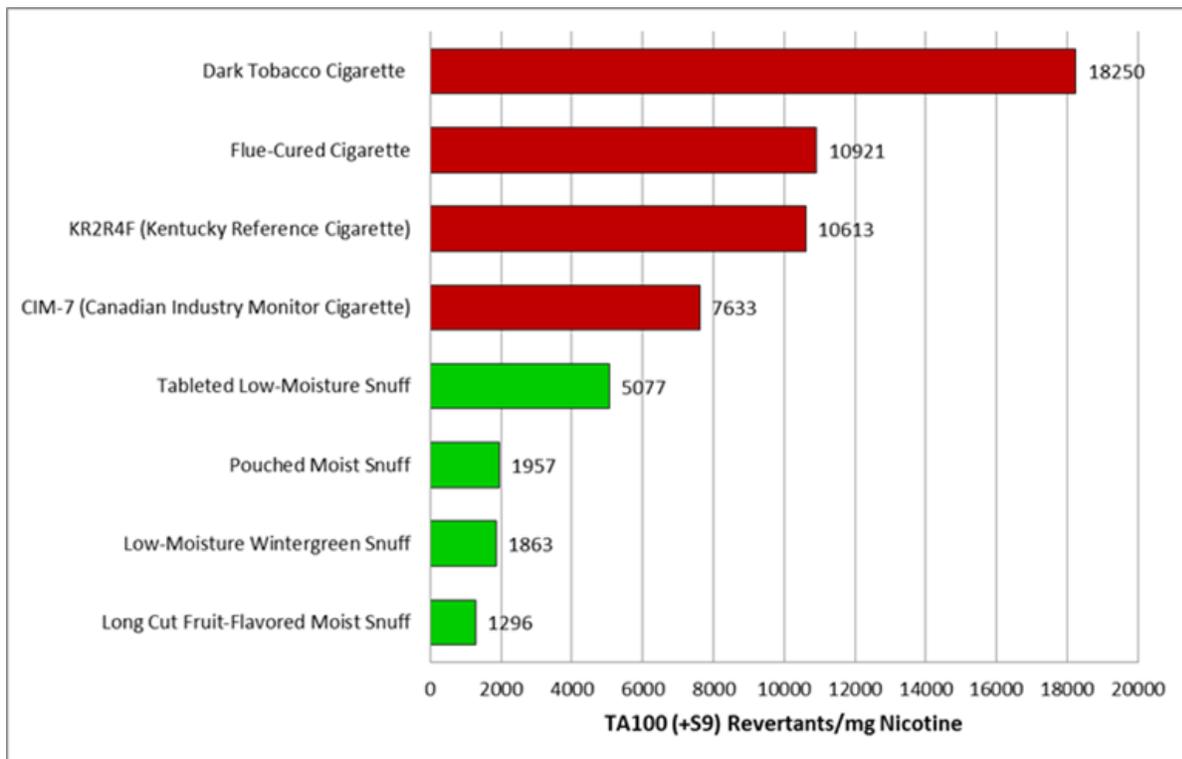
(ST) liquid extracts. The commercial products analyzed included Marlboro Gold cigarettes, Copenhagen Snuff, and Marlboro Snus. Additionally, Kentucky reference cigarettes 3R4F and 1R5F were included in the evaluation. The authors reported that when cigarette smoke TPM and smokeless tobacco extract were tested at concentration ranges that would approximate those experienced by users of either product, the smokeless tobacco extracts exhibited substantially lower toxicity compared with smoke condensate. In the case of the Ames mutagenicity test, the specific activity for the combustible cigarette examined was found to be 1600-1850 and 500-750 revertants/mg WTPM (wet total particulate matter) for strains TA98 and TA100, respectively. For these same strains, the ST products did not elicit an average response greater than 2 revertants/mg of extract. Similarly, the cytotoxicity of cigarette smoke TPM measured using the Neutral Red Uptake assay was found to be significantly higher than that of the ST extracts, which did not elicit a response significantly greater than that seen for solvent controls at the levels tested.

Song *et al.* have recently reported product chemistry analyses and *in vitro* genotoxicity and cytotoxicity findings for a variety of conventional U.S. moist snuff products, U.S. and Swedish snus products, Kentucky 2R4F Reference cigarettes and the Kentucky Reference smokeless products 2S1, 1S2 and 2S3 (Song *et al.* 2016). The authors also stated that data from the Kentucky 2R4F Reference cigarette were collected, but these findings were not reported in the paper. The evaluated U.S. snus products included Camel Snus Frost, Camel Snus Spice and Camel Snus Original varieties in addition to four Marlboro Snus varieties. Three Swedish and two South African smokeless tobacco products were also evaluated. Ames mutagenicity assays (in strain TA102 + S9 only), Neutral Red Uptake cytotoxicity assessments (CHO cells), and *in vitro* micronucleus assays (CHO cells) were conducted with DMSO extracts of the smokeless tobacco products. No significant bacterial mutagenicity was detected for the tested U.S. snus products, which included two Camel Snus varieties. The U.S. snus products were found to be slightly more cytotoxic than the tested conventional moist snuff products, inducing a maximum of 50% cell lethality vs. 34.8% lethality, respectively, under the assay conditions. No difference between the tested U.S. snus products and conventional moist snuff products was seen in the *in vitro* mammalian cell micronucleus assay. The authors concluded that all of the tested smokeless tobacco products had minimal *in vitro* toxicity.

The genotoxicity and cytotoxicity of both combusted and smokeless tobacco products may vary among the varieties of different products within these broad categories. A 2007 report by Rickert and co-workers provides a comparative analysis of mutagenic activity from a large variety of tobacco products (Rickert *et al.* 2007; see Figure 6.1.3-1 below). In the Ames bacterial mutagenesis assay, no moist snuff products were mutagenic in the frame-shift tester strain TA98, a strain in which cigarette smoke condensates show high mutagenic activity and a strong dose-response in the presence of S9 metabolic activation (Rickert *et al.* 2007; DeMarini 2004). Some response to moist snuff extracts was observed using the base pair-substitution test strain TA100, and so a comparison was made among a variety of tobacco products, normalized by nicotine content, using that strain (Rickert *et al.* 2007). As seen in Figure 6.1.3-1 below, moist snuff products exhibit only a fraction of the mutagenic activity of cigarette smoke. These findings are consistent with an expectation of lower systemic mutagen exposure from

smokeless tobacco as it is used by consumers, as is confirmed by the absence of mutagenic activity in urine of users of the U.S. smokeless products Copenhagen, Skoal Bandits-Wintergreen and Hawken-Wintergreen (Benowitz *et al.* 1989), or Swedish smokeless tobacco products (Curvall *et al.* 1987).

Figure 6.1.3-1: Smokeless tobacco is less mutagenic than combusted tobacco products when analyzed with the Ames bacterial mutagenesis assay (from Rickert *et al.* 2007)



In summary, the available published *in vitro* studies of smokeless tobacco provide a consistent body of evidence in documentation of its comparatively low cytotoxic and genotoxic hazard relative to cigarette smoke. It was stated some years ago that *in vitro* toxicology methods for smokeless tobacco are “useful for screening toxicity, but have not been validated for predicting human health risks” (Johnson *et al.* 2009). Notably, however, in the case of smokeless tobacco products there is an extensive body of extant epidemiologic data demonstrating their substantially lower risks to human health relative to cigarette smoking. This unusual circumstance presents an opportunity to evaluate the utility of *in vitro* methods as predictors of human risks in a manner that reverses the convention by which controlled experimentation in the toxicology laboratory is pursued to predict risk outcomes in the human population. Data from U.S. and Swedish epidemiology studies (Lee and Hamling 2009a; Colilla 2010) has been reported to show that smokeless tobacco use is associated with relatively low risks for cancer and other diseases (Lee 2013a). This is in stark contrast to epidemiology findings consistently reported for combusted tobacco products such as cigarettes (IARC 2004, USDHHS 2004). The epidemiology of smokeless tobacco products used in the U.S. and Scandinavia provides confirmation of the risk differential relative to smoking that would be predicted from the

results of *in vitro* toxicology studies of those products (Siddiqi *et al.* 2015). Though smokeless tobacco has not been as extensively studied as has cigarette smoke with *in vitro* test systems, the findings from diverse, independent laboratories around the world are quite consistent in reporting that smokeless tobacco has minimal cytotoxic and genotoxic activities relative to cigarettes. These data are also consistent with and complement the epidemiology discussed in Section 6.1.1 with respect to differential risks between smokeless tobacco and cigarettes as they are used by consumers.

6.1.3.4 Overview of RJRT *in vitro* genotoxicity and cytotoxicity studies

RJRT has performed a series of *in vitro* genotoxicity and cytotoxicity studies with the subject Camel Snus products that are aligned with the rationale presented below. The detailed study overviews presented below include discussion of portions of experimental research investigations (Studies M97, M100 NRU, M100 MN, M125) as well as tests performed in accordance with GLP provisions (Studies M194A-GLP, M194B-GLP). Each of the studies included statistical comparisons of findings for Camel Snus to those of concurrently-tested combustible cigarettes. These studies are itemized in Table 6.1.3-2 below:

Table 6.1.3-2: RJRT *in vitro* Genotoxicity and Cytotoxicity Studies

Study	Title	Camel Snus Style(s) Tested
M97 [Ames]	Toxicology of Smokeless Tobacco Products: Bacterial Reverse Mutagenicity	Frost
M100 NRU	Toxicology of Smokeless Tobacco Products: Neutral Red Cytotoxicity	Frost
M100 MN	Toxicology of Smokeless Tobacco Products: <i>In Vitro</i> Micronucleus Assays	Frost
M125 [SCE]	Toxicology of Tobacco Products: Sister Chromatid Exchange Genotoxicity	Frost
M194A-GLP [Ames]	Determination of Mutagenic Response of Camel Snus and Other Tobacco Products	Frost, Frost Large, Mellow, Mint, Robust, Winterchill
M194B-GLP [NRU]	Determination of Cytotoxic Response of Camel Snus and Other Tobacco Products	Frost, Frost Large, Mellow, Mint, Robust, Winterchill

6.1.3.4.1 Rationale for the selection of a battery of Ames Salmonella bacterial strains to evaluate and compare the mutagenicity of Camel Snus and major brands of cigarettes and smokeless tobacco

The battery of Ames *Salmonella* strains used in this evaluation included TA1535, TA1537, TA98, TA100 and TA102; each with and without an exogenous metabolic activation system comprising induced rat liver S9 and appropriate cofactors. These tester strains constitute the battery recommended by regulatory and scientific bodies for the evaluation of tobacco products, chemicals and pharmaceuticals ([Health Canada 2004](#); [OECD 1997](#); [FDA 2012a](#)). The study methods were a contemporary iteration of the original procedure ([Ames et al. 1975](#); [Maron and Ames 1983](#)) that has generated a large database confirming its ability to detect genetically active compounds of most chemical classes with around 60 to 80% sensitivity and specificity ([Gatehouse et al. 1990](#)). Each tester strain and activation system condition responds to different classes of chemical compounds, so a battery of tester strains is particularly appropriate for the testing of complex mixtures. Although not all Ames tester strains are known to respond to cigarette smoke preparations (*e.g.*, TA1535 and TA102, [Belushkin et al. 2014](#)), this full battery of strains was employed to establish a complete characterization of any potential mutagenic properties of any of the Camel Snus products that are the subject of this Application.

A considerable, international body of such *in vitro* mutagenicity data is available (*see Johnson et al. 2009*, Table 14) to show that tested cigarette smoke condensates typically produce:

- robust, dose-responsive mutagenic activity in strains TA98 and TA100 in the presence of S9, and modest activity in TA98 and TA100 without S9;
- modest to moderate activity in TA1537 with or without S9; and
- very little, inconsistent or no activity at all in TA1535 or TA102 with or without S9.

The Ames *Salmonella*/microsome mutagenicity assay has been adapted in recent years to accommodate testing of whole smoke aerosols or the gas/vapor phase of the smoke aerosol, and several authors have reported additional mutagenic activity, especially in strains TA98 and TA100 without S9, for the gas/vapor phase ([Aufderheide and Gressmann 2008](#); [Thorne et al. 2015a](#)). Strain TA102, which has been found to detect the mutagenicity of some crosslinking, reactive oxidative mutagens and carbonyls in isolation (*e.g.*, hydrogen peroxide, formaldehyde), has not proven to reliably detect mutagenicity from exposures to either TPM, whole smoke, or the gas/vapor phase of cigarette smoke.

The methods for whole smoke aerosol exposures are not presently refined or standardized to the extent that those for the testing of cigarette smoke condensates have been in over 40 years of research and testing. Efforts to establish such standardization are underway ([CORESTA 2007](#), [CORESTA 2015](#)). It is apparent from studies to date, however, that the substantial majority of mutagenic activity that is demonstrated by cigarette smoke *in vitro* resides in the smoke particulate phase, as do the majority of harmful and potentially harmful smoke constituents.

Since the present evaluations considered the activity of only the smoke particulate matter from the tested cigarettes, the differences in mutagenicity between extracts of the Camel Snus products and the cigarette smoke condensates likely under-represent the magnitude of differences between the Camel Snus extracts and that of the whole smoke aerosol that includes consideration of any mutagenic gas/vapor phase constituents.

(b) (4)



FDA has properly advised that “[t]here are several criteria for determining a positive result, such as a concentration-related increase over the range tested and/or a reproducible increase at one or more concentrations in the number of revertant colonies per plate in at least one strain with or without metabolic activation system. Biological relevance of the results should be considered first. Statistical methods may be used as an aid in evaluating the test results. However, statistical significance should not be the only determining factor for a positive response. A test substance for which the results do not meet the above criteria is considered nonmutagenic in this test” (FDA Redbook 2000). FDA further elaborates on its criteria for assessing *in vitro* findings in its Guidance for Industry for the assessment of biological relevance of *in vitro* genotoxicity testing of pharmaceuticals (FDA 2012a, pp. 14-15). Similarly, ICH guideline S2 (R1) on Genotoxicity testing and data interpretation states:

“Examples of results that are not considered biologically meaningful include:

- i. Small increases that are statistically significant compared with the negative or solvent control values but are within the confidence intervals of the appropriate historical control values for the testing facility
- ii. Weak/equivocal responses that are not reproducible”

(ICH 2011, p. 15).

FDA’s perspective is therefore consistent with those of other international regulatory bodies, and RJRT concurs that consideration of the biological relevance of statistical findings of mutagenicity is appropriate in order to develop the most informative perspective on *in vitro* test results.

6.1.3.4.2 Rationale for the selection of the micronucleus assay to evaluate and compare the genotoxicity of Camel Snus and cigarette smoke

The *in vitro* micronucleus assay detects genotoxins that act through disruption of chromosomal structure directly, or through effects on chromosomal replication in dividing cells. Membrane-enclosed chromosomal fragments (micronuclei) resulting from such disruptions may be readily counted visually or electronically in treated cells or in *ex vivo* tissue samples. The potency of a variety of test chemicals in inducing micronuclei in mammalian cells *in vitro* has been shown to correlate well with their genotoxicity in other *in vitro* assay systems, as well as with their tumorigenicity *in vivo* (Wills *et al.* 2016a; Wills *et al.* 2016b). Concurrent testing in a battery comprising the Ames *Salmonella* mutagenicity assay and the *in vitro* micronucleus assay has been demonstrated to have a very high concordance with results from chronic cancer bioassays (Kirkland *et al.* 2011). The authors of this formal analysis of the performance of this *in vitro* battery in detecting rodent carcinogens/genotoxins listed in a database of 962 compounds have stated “there is no convincing evidence that any genotoxic rodent carcinogens or *in vivo* genotoxins would remain undetected in an *in vitro* test battery consisting of Ames + [the *in vitro* micronucleus assay]” (Kirkland *et al.* 2011).

6.1.3.4.3 Rationale for the selection of the *in vitro* sister chromatid exchange assay to evaluate and compare the genotoxicity of Camel Snus and cigarette smoke

Of the available assays that have been used to assess the genotoxicity of cigarette smoke in human smokers, some (*e.g.*, *hprt* mutation frequency and spectra in peripheral lymphocytes; Curry *et al.* 1999) have proven inconsistent in demonstrating effects attributable to smoking (Albertini *et al.* 1988; Sala-Trepat *et al.* 1990). This is in contrast to measures of sister chromatid exchanges (SCE), which have been shown to be reliably detected as being elevated in peripheral lymphocytes from smokers in several studies (*e.g.* Livingston and Fineman 1983; Sarto *et al.* 1987). Additionally, an *in vitro* SCE assay performed in V79 cells in the presence of an induced rat liver S9 metabolic activation system has been reported to respond in a dose-dependent

manner to NNK ([Zimonjic et al. 1989](#)), suggesting its potential utility in comparative testing of mixtures containing this tobacco-specific nitrosamine. The measurement of SCEs in response to cigarette smoke and smokeless tobacco extracts *in vitro* therefore represents an endpoint that may be informative with respect to exposures that occur among human populations that use tobacco products.

6.1.3.4.4 Rationale for selection of the mammalian cell neutral red uptake cytotoxicity assay to evaluate and compare the cytotoxicity of Camel Snus and cigarette smoke

The potential of cigarette smoke to kill mammalian cells is generally accepted to have a central role in the etiology of adverse smoking-related health outcomes; notably cancer and respiratory disease, and possibly certain cardiovascular conditions ([USDHHS 2010](#)). The majority of this etiologic significance derives from smoke-induced local and systemic inflammation, as well as restorative epithelial hyperplasia (*i.e.*, a primary mechanism of tumor promotion) that occurs consequent to acute cell lethality in smoke-exposed tissues of the respiratory tract.

Numerous mammalian cell cytotoxicity assays, including neutral red dye uptake, LDH release, kenacid blue binding, MTT formation, XTT formation, acid phosphatase activity, sulforhodamine B binding and resazurin binding, have been used to evaluate the cytotoxicity of cigarette smoke or its condensates. Among these, the mammalian cell neutral red uptake assay has proven to be the most sensitive, particularly under conditions of moderately extended exposure durations that encompass the time ranges typical of smokeless tobacco usage ([Putnam et al. 2002](#); [CORESTA IVTF 2004](#)).

The gas/vapor phase of cigarette smoke has been demonstrated to account for the majority (~65%) of the cytotoxicity induced by that complex aerosol in an advanced *in vitro* apparatus capable of exposing cultured mammalian cells at the air/liquid interface ([Thorne et al. 2015b](#)). Since the present evaluations considered the activity of only the cigarette smoke particulate matter from the tested cigarettes, the differences in cytotoxicity between extracts of the Camel Snus products and the cigarette smoke condensates, highly statistically significant as they are, likely under-represent the magnitude of differences between the Camel Snus extracts and that of the whole smoke aerosol that includes consideration of cytotoxic gas/vapor phase constituents.

6.1.3.4.5 Rationale for selection of comparator products

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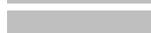
The Institute of Medicine has advised that comparisons of a candidate MRTP to leading brands of existing products that are “most commonly used by consumers are likely to provide a good comparison for products that claim to demonstrate reduced health risk” and further that “a set of products that account for a significant portion of the market could capture subgroups of interest.” The use of leading brand comparators “increases the likelihood that the findings will have broader applicability to the population, which is crucial given the public health standard against which MRTPs are evaluated” (IOM 2012, p. 237). The IOM also advised that comparisons both across product classes (here, Camel Snus vs. leading brand cigarettes) and within product classes (here, Camel Snus vs. other leading brands of smokeless tobacco products) may be appropriate (IOM 2012, pp. 237-238). RJRT concurs with this rationale for selection of comparison products, and has pursued both of these recommended comparisons in the *in vitro* testing of the Camel Snus products that are the subject of this Application.

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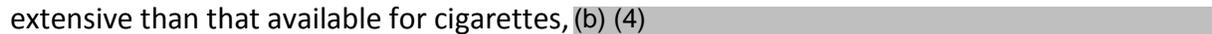


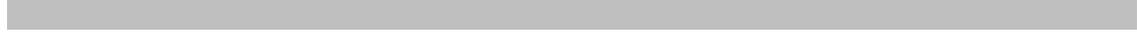
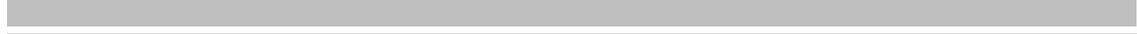
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. Cigarettes of the Kentucky reference series have been widely used in academic, governmental and industry laboratories around the world to provide common points of comparison among different laboratories for analytical chemistry and toxicology studies. Cigarettes of the Kentucky reference series have been shown to be broadly representative of commercial cigarettes from the U.S. market in terms of their smoke chemistry (Chepiga *et al.* 2000) and Ames mutagenicity (Steele *et al.* 1995), and their smoke chemistry and genotoxicity have been periodically compared across different production cycles (Roemer *et al.* 2012).

The volume of published *in vitro* toxicology data on smokeless products of all types is less extensive than that available for cigarettes, (b) (4)




(b) (4)



The 2S3 Research Moist Snuff and CRP1 Reference Snus were developed and distributed by North Carolina State University as reference products to facilitate comparative analyses, testing and research across laboratories in a manner akin to that of the University of Kentucky Research Cigarette series ([NCSU 2015](#)). These smokeless products and others of the series are commonly employed as points of reference and comparison by research laboratories to monitor the performance of analytical and other testing procedures.

(b) (4)



6.1.3.5 Genotoxicity studies of Camel Snus [bacterial mutagenesis, mammalian cell micronuclei and sister chromatid exchanges] relative to cigarette smoke

Per the rationale presented above, the series of *in vitro* toxicology evaluations of Camel Snus that are submitted in support of this Application are individually listed and discussed below. These included both initial investigations conducted to refine dose selection and testing conditions, as well as later studies performed in accordance with applicable provisions of Good Laboratory Practices guidelines ([21 CFR Part 58](#), as amended 21 May, 2002).

6.1.3.5.1

M97: Toxicology of Smokeless Tobacco Products: Bacterial Reverse Mutagenicity

Purpose

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Design

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Results

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Conclusions

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6.1.3.5.2 **M100 MN: Toxicology of Smokeless Tobacco Products: *In Vitro* Micronucleus Assay**

Purpose

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Design

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(b) (4)



Results

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Conclusion

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6.1.3.5.3

M125: Toxicology of Tobacco Products: Sister Chromatid Exchange Genotoxicity

Purpose

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Design

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Results

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Conclusions

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6.1.3.5.4 M194A-GLP: Determination of Mutagenic Response of Camel Snus and Other Tobacco Products

Purpose

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Design

(b) (4)



(b) (4)



Assay Methodology

(b) (4)



Data Analysis

(b) (4)



Results

(b) (4)



(b) (4)

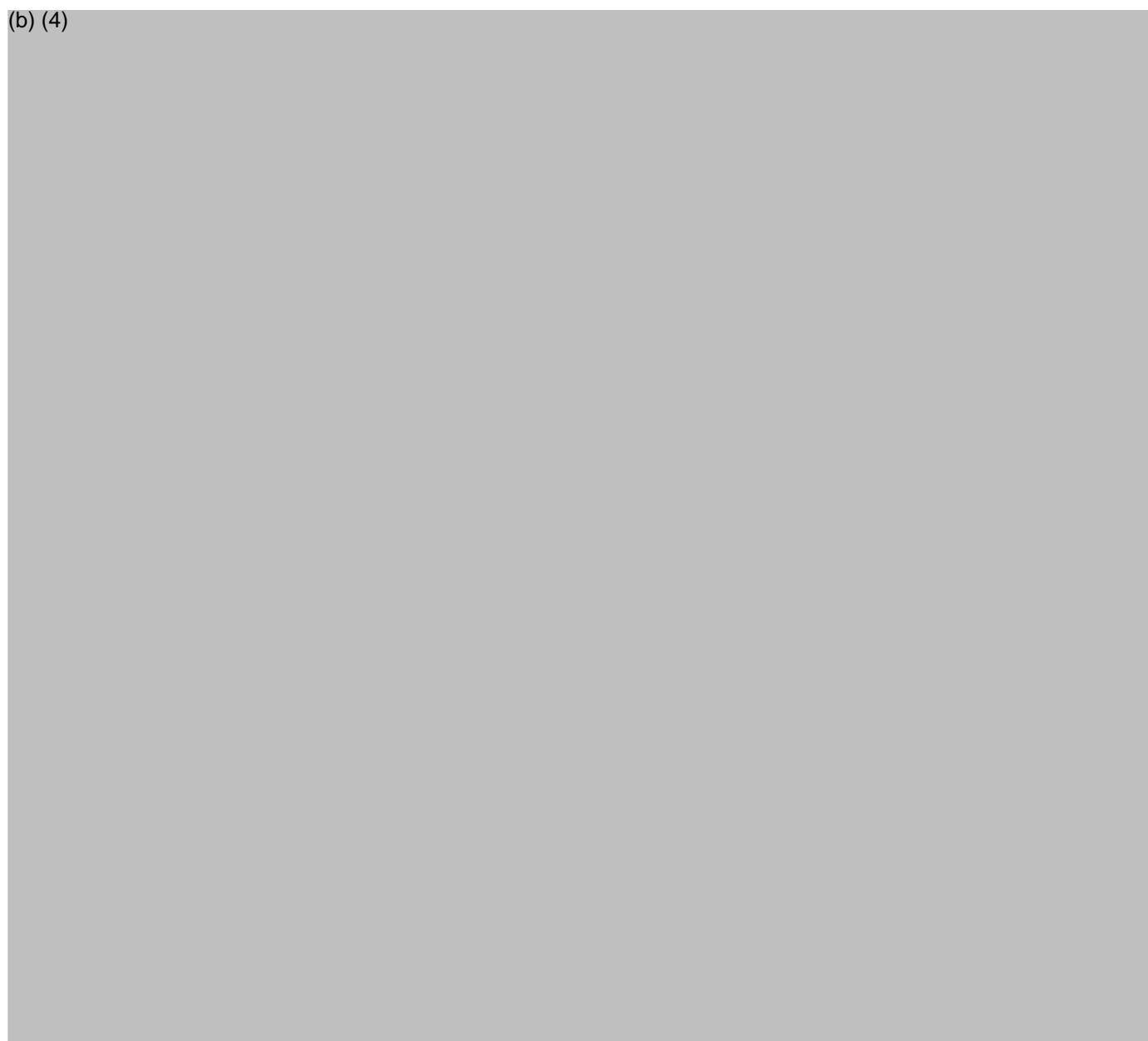
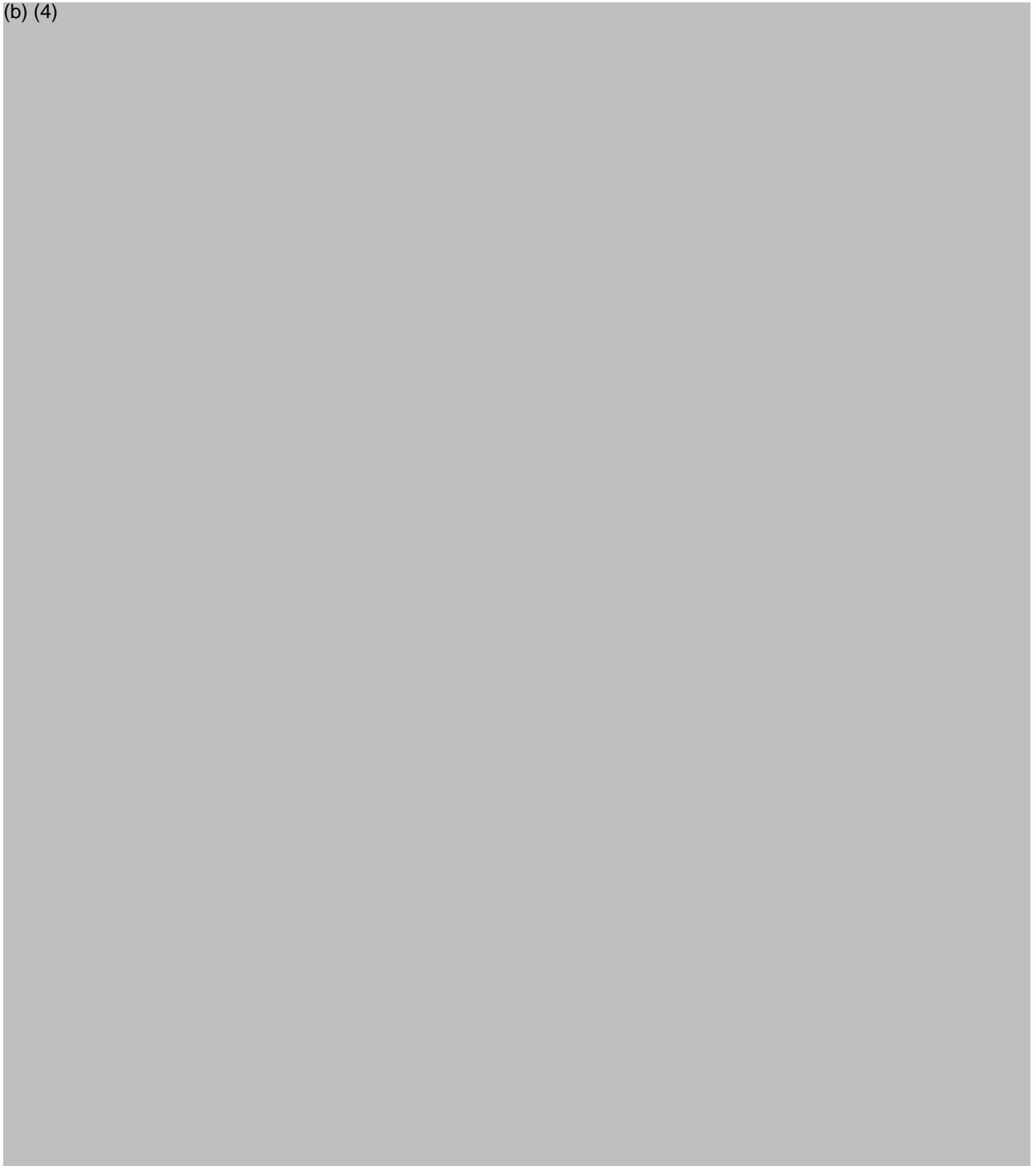


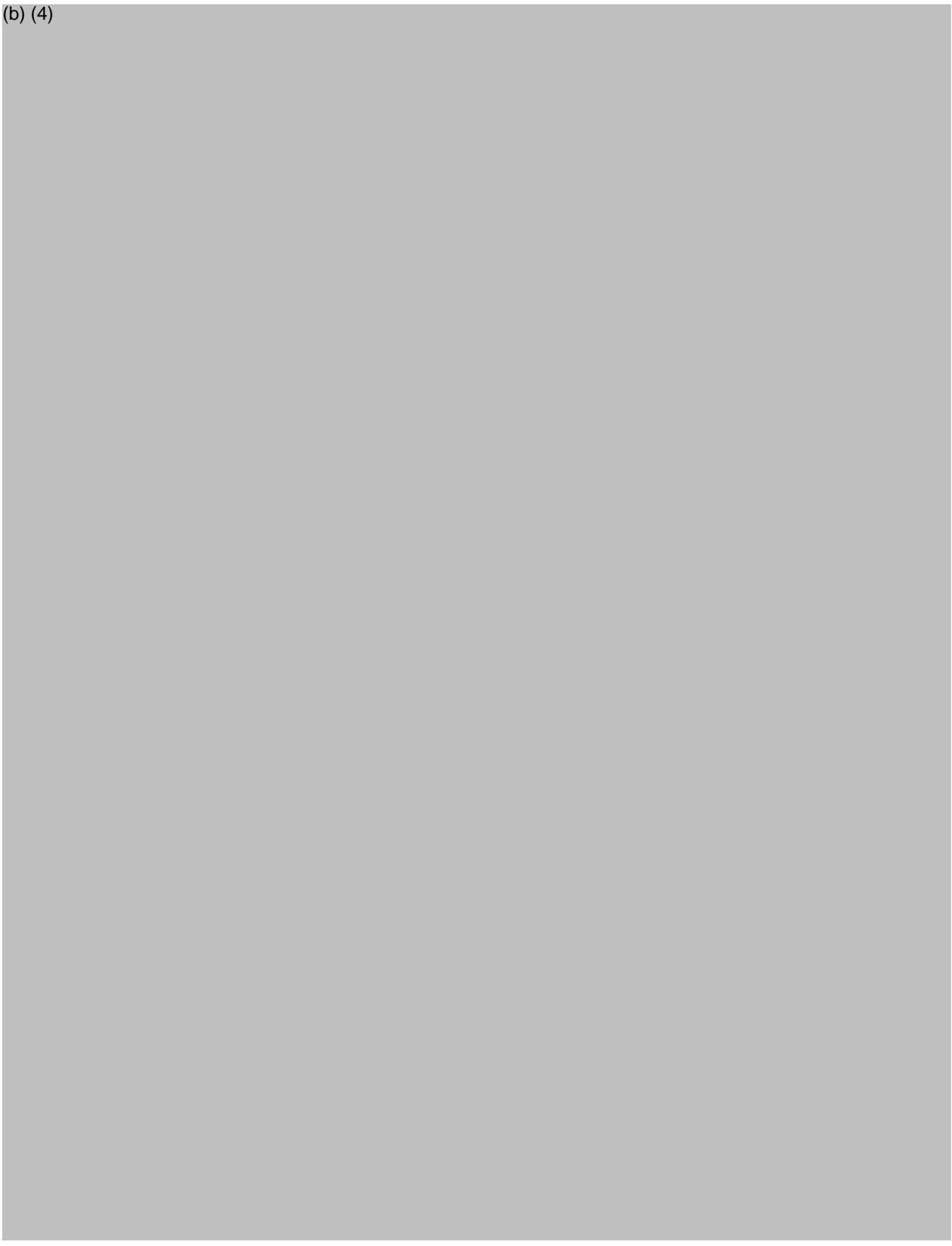
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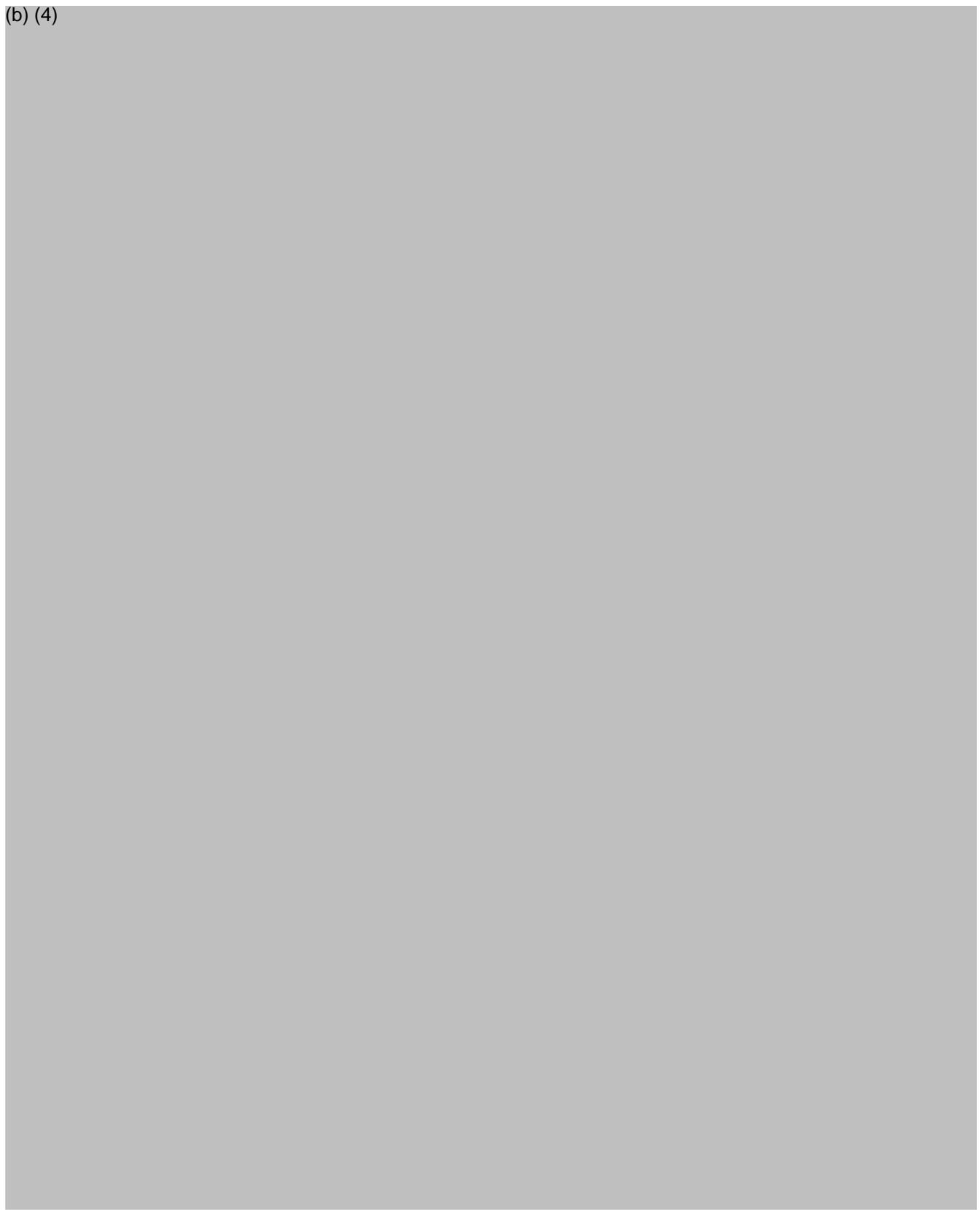
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Discussion

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Conclusions

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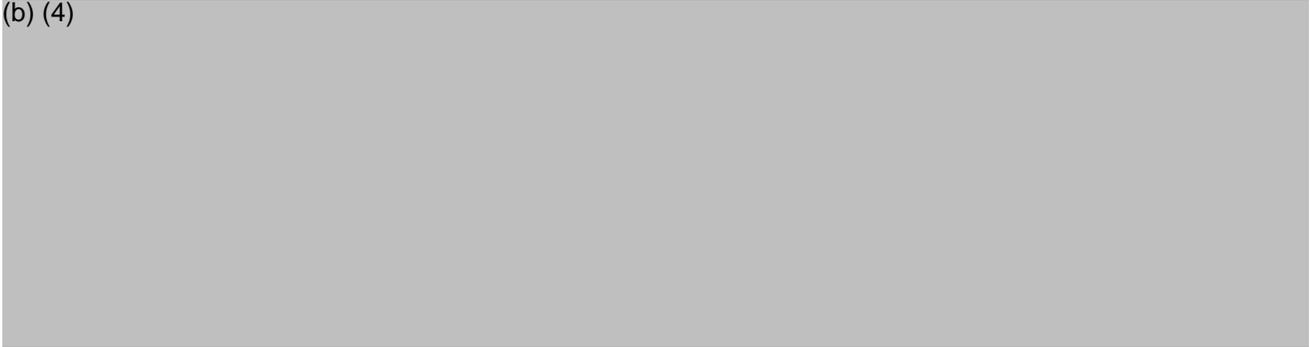


6.1.3.6 Cytotoxicity studies of Camel Snus [Neutral Red Uptake] relative to cigarette smoke

6.1.3.6.1 **M100 NRU: Toxicology of Smokeless Tobacco Products: Neutral Red Cytotoxicity**

Purpose

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Results

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Conclusions

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6.1.3.6.2 **M194B-GLP: Determination of the Cytotoxic Response of Camel Snus and Other Tobacco Products**

Purpose

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Design

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Assay Methodology

(b) (4)



Data Analysis

(b) (4)



(b) (4)



Results and Discussion

(b) (4)



Table 6.1.3-3: (b) (4)



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(b) (4)



Table 6.1.3-4: (b) (4)



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Table 6.1.3-5: (b) (4)



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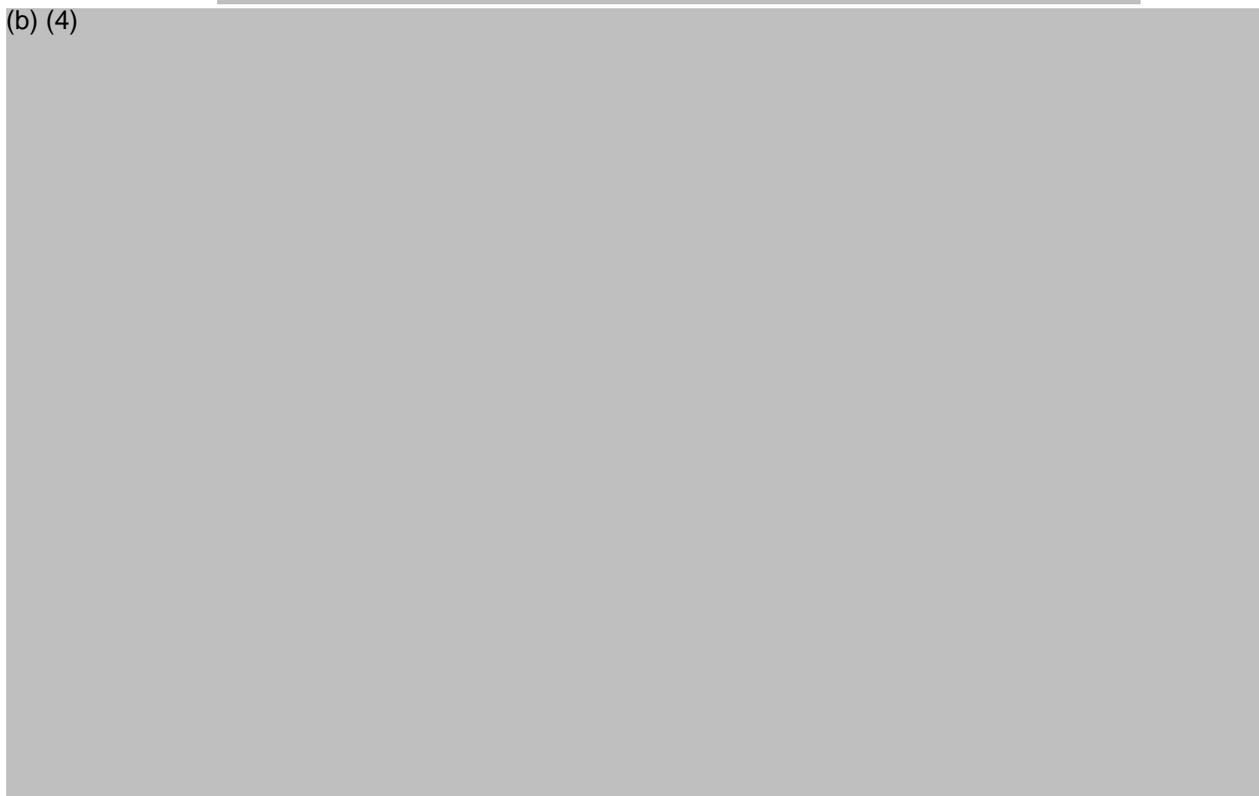
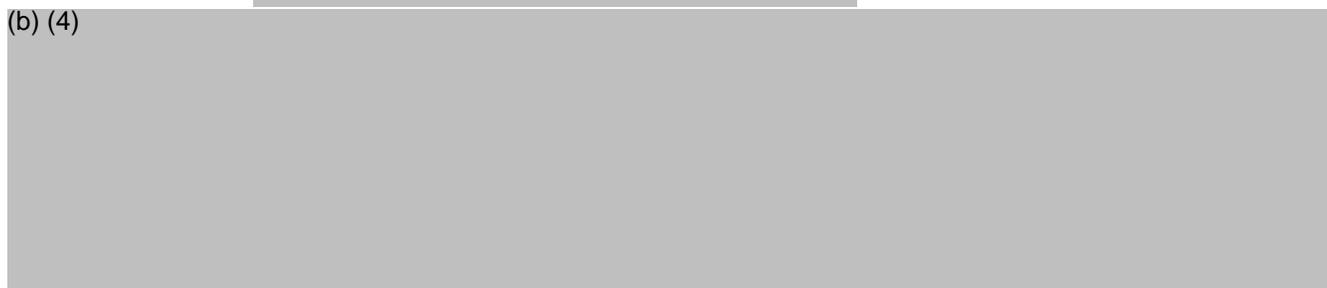


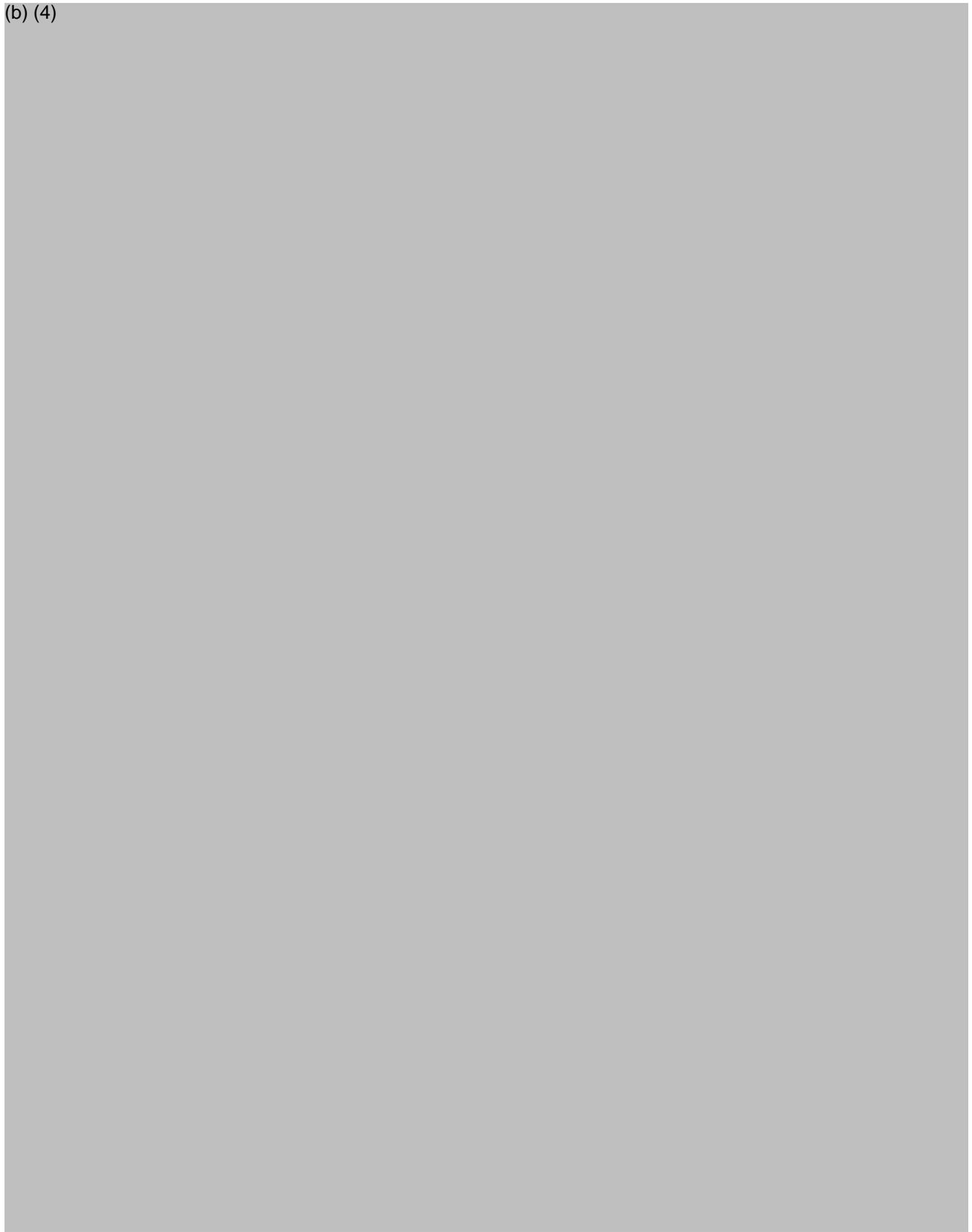
Table 6.1.3-6: (b) (4)



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6.1.3.7

Conclusions

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(b) (4) These findings are consistent with an expectation of lower risks from Camel Snus than from cigarette smoking for diseases in which cytotoxicity may play an etiologic role.

These *in vitro* data complement and concur with data from product analyses, *in vivo* toxicology evaluations, clinical investigations and epidemiology studies that are presented elsewhere in this Application, and contribute to a toxicology-based foundation of biological plausibility for the reduced risks for cancer and other diseases that have been reported for smokeless tobacco users relative to cigarette smokers. Further, the *in vitro* findings for all six of the subject Camel Snus brand styles are consistent with an expectation that any genotoxic and cytotoxic properties they may have are either similar to, or less than those of other U.S. smokeless tobacco products that have been associated with substantially lower disease risks relative to cigarettes in epidemiologic studies of U.S. populations.