

6.1.4 In Vivo Toxicology Studies

Scientific studies using laboratory animals are key scientific components of FDA oversight across most of its regulated product sectors, and are specifically recommended in FDA's Draft Guidance for Modified Risk Tobacco Product Applications ([FDA MRTPA Draft Guidance 2012](#)). Whereas both FDA and regulated product manufacturers support ongoing effort to reduce, replace and refine (the "3 Rs") *in vivo* experimentation to minimize the use of living animals in nonclinical safety assessments, at the present time such studies continue to serve an important role in regulatory science as a link between the information generated by analytical laboratory and *in vitro* toxicology studies and by human clinical and epidemiologic investigations.

The following sections briefly review the available *in vivo* literature, demonstrating that in laboratory animals: (1) exposure to cigarette smoke and smoke condensates promotes tumor formation, cardiovascular disease, significant respiratory tract histopathology and impaired respiratory function; (2) chronic dietary or topical oral exposures to smokeless tobacco of the types used in the U.S. and Sweden have demonstrated no consistent, serious toxicity or carcinogenicity in the oral cavity or other sites; and (3) the Camel Snus tobacco blend, as well as aqueous extract of that blend, exhibits low systemic toxicity and no carcinogenicity following chronic, high-level dietary administration to rats. The consistent findings of markedly lower *in vivo* toxicity and carcinogenicity of smokeless tobacco ("ST") products are concordant with epidemiologic evidence from the U.S. and Sweden that shows smokeless tobacco users are at lower risk for oral and lung cancer development, cardiovascular disease, and serious respiratory disease when compared to cigarette smokers.

The *in vivo* studies sponsored by RJRT in support of this Application were all performed by fully-accredited, independent, extramural testing facilities. All *in vivo* studies were performed in accordance with applicable U.S. laws, regulations, and policies governing animal testing, as is further specified in the final research reports submitted with this Application.

6.1.4.1 Rationale for the use of *in vivo* evaluations in the comparative evaluation of tobacco products

The International Agency for Research on Cancer has stated that "[a]ll known human carcinogens that have been studied adequately in experimental animals have produced positive results in one or more animal species" ([IARC 2007b, p.17](#)). It is well understood, however, that laboratory animals may exhibit metabolic and other physiological differences that can complicate the direct, quantitative extrapolation of their findings to predictions of human risks. In light of their inherent physiological and methodological limitations, *in vivo* studies are of greatest value in qualitative determinations of potential hazards, as extrapolation of results from *in vivo* studies to humans falls short of perfect concordance ([Stratton et al. 2001](#); [IOM 2012](#)). That said, the intact digestive, metabolic, endocrine, respiratory and neurological systems of living mammalian laboratory animals provide the best current means to qualitatively identify chemicals and mixtures that may have either therapeutic, nutritional, toxic or carcinogenic potential in humans ([SOT 1999](#)). Quantitative (potency) comparisons among test

articles are also possible, within the constraints common to all animal studies, with appropriate *in vivo* testing strategies that compare test outcomes against those of other chemicals or mixtures of interest that have been similarly evaluated.

Scientific investigations in laboratory animals to advance understanding of the potential toxic and carcinogenic properties of tobacco and tobacco smoke began in earnest around the middle of the 20th century. Prior to that time, research into tobacco and tobacco smoke had been sporadic, resulting in a relatively small number of published reports developed from the crude *in vivo* toxicology methods of the day. Many approaches were undertaken with different animal species, various tobacco types, and different methods of tobacco test material preparation and dosing. The results of the early studies were mostly negative or were conducted with too few animals to be regarded as noteworthy. It was not until 1953 that the first significant results were obtained by Dr. Ernst Wynder and colleagues, who reported that mice painted chronically with tobacco smoke condensate developed excess skin tumors compared to untreated mice (Wynder *et al.* 1953). That observation prompted a period of intense study of cigarette smoke toxicity in laboratory animals that continues to the present day.

6.1.4.1.1 Rationale for consideration of subchronic rather than chronic *in vivo* cigarette smoke exposure studies in the comparative evaluation of cigarettes and smokeless tobacco products

Subchronic evaluations of inhaled cigarette smoke in laboratory animals have proven to be informative in terms of modeling certain aspects of the mechanisms that underlie the development of serious, smoking-related respiratory diseases such as COPD and lung cancer. Many of the inflammatory and histopathologic changes seen in cigarette smoke exposure studies of 28 or 90 days duration in laboratory animals are qualitatively similar to those observed in human smokers (Higuchi *et al.* 2004; Friedrichs *et al.* 2006). Subchronic cigarette smoke inhalation studies in rats, typically of 90-days duration, have been extensively used to compare among cigarettes in evaluating the potential of cigarette construction and compositional variables to affect smoke-induced systemic toxicity and respiratory histopathologic responses (Heck *et al.* 2002; Vanscheeuwijck *et al.* 2002; Higuchi *et al.* 2004; Theophilus *et al.* 2007). Such studies reliably produce respiratory tract hyperplasia, metaplasia, inflammatory cell influx and tissue damage similar to that observed both in longer-term, chronic smoke exposure studies in rodents and in chronic human smokers. Systemic inflammation, manifested as increases in inflammatory signaling molecules and indices of oxidative stress, are reliably demonstrated in contemporary subchronic smoke exposure experimentation. Functional and biomarker changes analogous to those observed in human smokers who are at elevated risks for cardiovascular disease are likewise demonstrable in subchronic rodent inhalation studies with cigarette smoke, manifested as adverse changes in lipid profiles, endothelial cell dysfunction and other measures (Lietz *et al.* 2013).

A considerable body of prior work has shown, however, that the extensive respiratory tract histopathology and other changes that are seen in subchronically-exposed rodents are generally reversible upon cessation of smoke exposure, and have therefore been characterized

largely as adaptive rather than premalignant changes (Burger *et al.* 1989). Chronic smoke exposure studies of extended durations of one year to the lifetime of experimental rodents (~ 2 years) consistently demonstrate very similar changes, but have proven to be largely unsuccessful in terms of reliably producing a significant incidence of lung cancers that resemble those that occur in human smokers. Chronic smoke inhalation studies have been attempted in a wide variety of rodent and non-rodent species, with similarly mixed and inconsistent findings (Coggins 1998; Coggins 2007). The failure of most chronic *in vivo* cigarette smoke inhalation studies to effectively model human lung carcinogenesis has limited their utility in research on smoking-related carcinogenesis (IOM 2012).

A variety of mouse strains, particularly those harboring genetic modifications intended to alter their susceptibility to smoke exposure (*e.g.*, rasH2 and ApoE^{-/-} mice), have shown positive responses to cigarette smoke exposure via inhalation that have included significant increases in lung tumors (Witschi *et al.* 2004; Curtin *et al.* 2004b; Hutt *et al.* 2005) and adverse effects on the cardiovascular system (Dong *et al.* 2010; Han *et al.* 2012; Lietz *et al.* 2013). Some inhalation studies using different rat strains have also reported modestly increased tumors of the respiratory tract following chronic cigarette smoke exposure (Dalbey *et al.* 1980; Mauderly *et al.* 2004). All of these reports, however, demonstrate significant limitations that constrain the general utility of smoke inhalation studies in animals as models for human cancers or to provide useful platforms for comparisons among different cigarettes. These limitations include the development of pulmonary tumors in very small percentages of the exposed animals (*e.g.*, conventional laboratory rat and mouse strains), the development of excessive numbers of spontaneous tumors in untreated control animals (*e.g.*, Strain A mice), and the production of tumors that do not reflect the histology of those seen in human smokers (Coggins 2010).

In summary, subchronic *in vivo* cigarette smoke inhalation studies have been shown to reliably demonstrate adverse effects of the respiratory and cardiovascular systems consistent with changes seen in chronic smokers who are at elevated risks for serious respiratory and cardiovascular diseases.

6.1.4.1.2 Rationale for consideration of *in vivo* mouse skin painting bioassays in evaluating the comparative tumorigenicity of cigarettes and other tobacco products

Whereas chronic cigarette smoke inhalation studies in rodents and other laboratory species have not been a robust and reliable means to produce lung tumors of the types seen in smokers, dermal application of cigarette smoke condensate and its fractions to mice, with subsequent observation of epithelial tumors, was first conducted in the 1950s (Wynder *et al.* 1953). Since that time a considerable number of peer-reviewed, published reports have consistently documented the dermal carcinogenicity of such cigarette smoke preparations in mice. The early *in vivo* dermal studies using this laboratory test system employed protracted cigarette smoke condensate treatments without prior treatments with tumor-initiating compounds, whereas the majority of later published investigations have described accelerated

tumor-promotion protocols using animals pre-treated with sub-threshold doses of genotoxic carcinogens to initiate the laboratory carcinogenesis process.

The topical treatments of rodents, usually mice, with cigarette smoke condensate or its fractions has continued in over 50 years of research to reliably produce very significant yields of benign papillomas and malignant carcinomas in the treated skin areas, often in 50% or more of the experimental animals (NCI 1980; Gaworski *et al.* 1999; Meckley *et al.* 2004). Mechanistic studies have shown that cigarette smoke condensate can act as a tumor initiator, a tumor promoter, a complete carcinogen and a co-carcinogen when applied topically to mice; demonstrating the versatility and utility of the mouse “skin painting” method in advancing the mechanistic understanding of the cancers caused by cigarette smoking (Hecht 2005).

Mouse skin painting studies, which typically entail repeated daily application of cigarette smoke condensate to the backs of mice for periods of 24 weeks to 1 year, produce substantial benign and malignant tumor yields in a dose-responsive manner; with increasing tumor incidence, increasing tumor multiplicity, and shorter tumor latency at higher doses of cigarette smoke condensate solutions. Whereas the dermal epithelium of the laboratory mouse does differ in some respects from the epithelia of the human oral and respiratory tract, it also shares several fundamental cellular and structural characteristics in common with those human tissues that are prominent sites of elevated tumor occurrence in chronic smokers.

The results of mouse skin painting studies are often reported in comparison to results obtained using a standardized reference cigarette (USDHEW 1979; NCI 1980; USDHHS 2010) or other control cigarette appropriate to the experimental design. Various dermal exposure protocols have repeatedly demonstrated that cigarette smoke condensate induces cytotoxicity, cellular proliferation, generation of damaging free radicals, and inflammation, all of which contribute to tumor promotion (*e.g.*, Curtin *et al.* 2004a). Mouse skin painting studies have also shown that cigarette smoke condensate may act at multiple steps in the carcinogenesis process (Hecht 2005). A body of experimental evidence has identified tumor promotion as the most significant contributor to cigarette smoke carcinogenesis in the mouse skin painting test system, and analyses of population data derived from several large population cohorts (Hazleton *et al.* 2005) has similarly indicated that tumor promotion is the primary etiologic mechanism of smoking-related lung cancer in humans as well. Topical studies demonstrating the carcinogenicity of cigarette smoke condensates may therefore be viewed as having certain mechanistic and histopathologic similarities to human cancers, albeit in the dermal epithelium of the mouse rather than in the respiratory epithelium that is the most prominent site of tumor development in smokers.

The Institute of Medicine, in its 2012 report on the comparative evaluation of tobacco products, has advised that mouse skin painting bioassays should be an essential component of the characterization of any combustible tobacco product due to their particular sensitivity to the polycyclic aromatic hydrocarbons, tumor promoters, and co-carcinogens that are found in cigarette smoke (see Table 3-4 and accompanying text in IOM 2012). This is in contrast to chronic cigarette smoke inhalation studies for which “the data are inconsistent” (IOM 2012).

In summary, a cumulative body of *in vivo* laboratory evidence demonstrates that topical applications of solutions of cigarette smoke condensates are unequivocally carcinogenic to the skin of laboratory mice, and such findings provide a point of reference to which the topical and systemic carcinogenic potential of smokeless tobacco or its extracts may be compared.

6.1.4.1.3 Rationale for *in vivo* oral feeding studies to assess the chronic toxicity and carcinogenicity of Camel Snus

Whereas *in vivo* evaluations of chronic toxicity and carcinogenicity are sometimes conducted by other routes of administration that resemble specific human exposure scenarios (*e.g.*, topical or inhalation exposure, biocompatibility assessments for implanted medical devices), the great majority of the chronic studies performed by the U.S. National Toxicology Program and those considered by IARC in its ongoing series of evaluations of human cancer risks have been conducted as feeding studies in laboratory rodents. Studies conducted by addition of the test article of interest to the standard diet of laboratory animals permits consistent dosing for the prolonged periods (years) required by contemporary standards for such testing. In certain instances, the dietary concentration of the tested material is adjusted over the course of the study to maintain dosing consistency as feed consumption changes over the course of the laboratory animals' lifespan. Rats are preferred over mice as a laboratory species for chronic toxicity and carcinogenicity bioassays for the practical advantages their larger body sizes offer with respect to dosing precision and larger organs that facilitate collection, weighing and histopathologic evaluation. Additionally, the rat is more similar to humans than the mouse in terms of its metabolic clearance of nicotine as well as in its responsiveness to acetylcholine receptor upregulation by this alkaloid ([Matta et al. 2007](#)).

The manner of use of smokeless tobacco (ST) by consumers presents a significant challenge to investigators seeking to replicate it in the *in vivo* laboratory. No robust and accepted rodent model of exposure to ST has been shown to reliably develop oral tumors with any significant frequency. Since chronic oral feeding studies of smokeless tobacco achieve both high local exposures to the oral cavity as well as systemic exposures to other potential target organs, and since Camel Snus is a product intended for partial ingestion by consumers, RJRT elected to sponsor a series of rodent feeding studies to elucidate the potential for oral and systemic toxicity and carcinogenicity by Camel Snus. The series culminated in a 2-year, GLP-compliant chronic toxicity/carcinogenicity study, the results of which show that ingested Camel Snus tobacco blend and aqueous extracts thereof produce no overt toxicological effects and no significant treatment-related tumor response in laboratory rats.

The *in vivo* studies of Camel Snus that were sponsored by the R.J. Reynolds Tobacco Company were aligned with the rationales presented above. These investigations, discussed subsequently, were conducted in the context of a body of prior, peer-reviewed, published research on both cigarettes and broadly similar smokeless tobacco products.

6.1.4.2 Published *in vivo* studies of cigarettes and smokeless tobacco

In vivo investigations of the toxicity and carcinogenicity of cigarette smoke and smoke extracts began in earnest in the 1950s, following sporadic reports in the earlier published literature. The body of published *in vivo* experimentation that has examined the potential toxicity and carcinogenicity of smokeless tobacco and smokeless tobacco extracts is less voluminous, and is discussed in more detail in the subsequent sections. Table 6.1.4-1 below lists representative, published *in vivo* studies of cigarette smoke, cigarette smoke condensate, smokeless tobacco and smokeless tobacco extracts, and the general findings that have been reported.

Table 6.1.4-1: *In vivo* bioassays used to evaluate cigarettes and smokeless tobacco in published studies

Test Methodology	Cigarette Smoke and Smoke Condensate	Smokeless Tobacco and Smokeless Tobacco Extracts	General findings
Subchronic and chronic rodent cigarette smoke inhalation	X		Severe histopathologic and inflammatory changes in the respiratory tract; systemic inflammation; few tumors
Chronic topical application to mouse skin	X		Robust carcinogenicity; numerous malignant epithelial tumors
Hamster cheek pouch insertion		X	No tumors
Rat surgical lip canal implantation		X	A few epithelial tumors in some studies
Oral swab studies		X	No tumors
Subchronic and chronic feeding		X	No significant or irreversible toxicity; no tumors in any organ or system
Transgenic rodent models	X	X	Snus caused gastric carcinoma in INS-GAS transgenic mice (incidence doubled by <i>H. pylori</i>); Tobacco smoke solutions and snus treatments of Elastase-IL-1 β transgenic mice yielded pancreatic histopathology, but no pancreatic tumors

6.1.4.2.1 Published studies of cigarette smoke and cigarette smoke condensate carcinogenicity

For the purposes of this Application, the vast literature on cigarette smoke *in vivo* toxicology is not reviewed anew; rather, the findings of selected and representative studies of cigarette smoke are cited and discussed for the purposes of comparing and contrasting them to findings from analogous published studies of smokeless tobacco.

The abundant *in vivo* scientific literature on the toxicity and carcinogenicity of cigarette smoke has been amply and repeatedly reviewed over recent decades. The International Agency for Research on Cancer provides a comprehensive review of these studies, and discusses their utility in developing its classification of tobacco smoke as a Group 1 carcinogen (IARC 2004). More recent studies in laboratory animals are reviewed in the 2010 U.S. Surgeon General's report (USDHHS 2010) in the context of establishing the biological plausibility of smoking as a cause of a number of serious diseases. Several volumes of the National Cancer Institute Tobacco Control Monographs series likewise provide reviews of the *in vivo* literature (see Sections 4.3 and 4.4 in NCI 1993 and Chapter 5 in NCI 2001). The Life Sciences Research Office, as a component of the LSRO Reduced-Risk Review Project, reviewed the *in vivo* cigarette smoke toxicology literature relating to neoplastic, respiratory and cardiovascular diseases; and advanced recommended approaches for the application of these and other methods to evaluate tobacco products having potentially reduced risks for users (LSRO 2007a; LSRO 2007b). The Institute of Medicine has also presented a compilation of *in vivo* and other studies of both smokeless tobacco and cigarettes (IOM 2012).

The cumulative body of published *in vivo* research findings are consistent with conclusions that cigarette smoke is demonstrably carcinogenic when applied to the skin of laboratory mice; subchronic and chronic cigarette smoke inhalation in laboratory rodents induces oxidative stress, unfavorable lipid profiles, inflammation and endothelial damage consistent with increased cardiovascular risk; and also induces multiple adverse respiratory tract histopathologic changes in laboratory animals consistent with those seen in serious chronic respiratory diseases such as COPD (USDHHS 2010).

6.1.4.2.2 Published studies of smokeless tobacco oral carcinogenicity

A number of experimental rodent models have been used to evaluate the oral carcinogenicity and toxicity of ST. These models have used smokeless tobacco exposures via hamster buccal pouches, surgically created oral canals in rats, oral swabbing, and feeding studies. The majority of historical *in vivo* studies examining the effects of ST have reported negative or equivocal results (see Table 8, p. 66-68, in SCENIHR 2008). Only 6 of the 28 studies discussed by SCENIHR produced any oral carcinomas at all, and those that did reported only 1-3 carcinomas among animal groups exposed chronically to smokeless tobacco. As noted by SCENIHR, a number of the available studies are poorly described, used insufficient numbers of animals, and did not comport with contemporary GLP standards. Additionally, the surgical intervention and local irritation imposed by the repeated introduction of the test materials in the buccal pouch and surgical lip canal models do not accurately reflect the manner of actual human ST product use,

and introduce certain confounding variables that complicate analyses of study findings, as discussed below. Only some studies where ST was inserted daily into surgically-created lip canals have demonstrated any carcinogenic potential in rats, and those results have been criticized due to the potential of tissue trauma associated with the surgical interventions to promote tumor development (SCENHIR 2008). Grasso and Mann, in a 1998 critical review of the body of published literature, concluded that “[d]espite the defects in some of the earlier studies, the sum total of this experimental work suggests that snuff is not carcinogenic to the oral mucosa of the hamster or the rat” (Grasso and Mann 1998). The conclusion of these authors reasonably summarizes the findings of the majority of studies that, overall, ST exhibits no consistent evidence of oral carcinogenicity in laboratory animals.

The subsequent sections discuss some of the primary published studies of smokeless tobacco that have used a variety of experimental approaches, and that have included gross and histopathologic evaluations of the occurrence of benign papillomas or malignant carcinomas, in animals exposed to smokeless tobacco or aqueous smokeless tobacco extracts.

6.1.4.2.2.1 Published hamster cheek pouch studies

Many early *in vivo* studies of ST used insertion of tobacco into the hamster cheek (buccal) pouches to simulate human ST “dipping” (Peacock and Brawley 1959; Peacock *et al.* 1960; Dunham *et al.* 1966; Antoniadis *et al.* 1984; Shklar *et al.* 1985; Summerlin *et al.* 1992). Although the pouches could retain test articles in the oral cavity for extended periods, approximating ST use in humans, such studies failed to produce any malignant tumors in any animals (SCENHIR 2008).

One study of ST in hamster buccal pouches did report oral tumor development when combined with viral exposure (Park *et al.* 1986). The authors administered regular deposits of ST with inoculations of herpes simplex virus (HSV-1 or HSV-2) for six months. Co-infection with HSV, a common human oral pathogen, is of potential significance due to several reported interactions with ST. These include inhibition of the HSV lytic reproductive cycle, a process suspected of involvement with the development of dysplastic lesions (Sand *et al.* 2014). Although half of the hamsters in the ST + HSV experimental groups developed invasive squamous cell carcinomas (21/40), none of the hamsters in the ST + mock inoculation experimental group exhibited any epithelial dysplasia or developed any tumors. The authors concluded that ST alone does not induce precancerous or neoplastic changes in hamster buccal pouches (Park *et al.* 1986). Critical commentaries on this experiment have included the point that epidemiologic data do not support extrapolation of HSV + ST rodent results to human users of ST, citing the rarity of snus-related oral cancers among Swedish users despite the common presence of HSV seropositivity in this population (ENVIRON 2010).

Alternative hamster models have been explored and reported, either mixing ST with feed in studies of 16 and 24 months duration (Dunham *et al.* 1974; Homburger *et al.* 1976) or placing a steel mesh cartridge containing ST inside the oral cavity for up to 12 months (Homburger 1971). No ST-related carcinogenic effects were reported in any of these studies, even though positive

controls such as polycyclic aromatic hydrocarbons did induce oral carcinomas (*see e.g., Homburger 1971*).

6.1.4.2.2.2 Published rat surgical lip canal studies

The Swedish dental surgeon Jan-Michael Hirsch first described a technique to carve an artificial surgical “canal” in the lower lip of rats. Test articles could then be repeatedly placed inside the canal for extended periods (*Hirsch and Thilander 1981*). A small test population of 2 male and 2 female rats was initially dosed with snus tobacco twice daily, five days a week, for 9 months. The dose administered was 1g/kg body weight, or approximately five times larger than the corresponding dose in humans (*citing Axell et al. 1976*). The rats showed no signs of severe toxic symptoms, but did show mild to moderate hyperplasia with hyperkeratosis. The authors acknowledged that the incidence of hyperkeratosis and slight dysplastic lesions was higher than reported in human studies (*citing Pindborg and Renstrup 1963; Roed-Petersen and Pindborg 1973; Axell et al. 1976*), which the authors attributed to the “amount of snus used, retention time, or species differences.” (*Hirsch and Thilander 1981*). The tissue trauma associated with the artificial avenue of exposure may also have contributed to the higher incidence of such lesions (*SCENIHR 2008*).

Hirsch and Johansson followed up with a second surgical lip canal study, administering snus (n = 42) or alkaline snus (n = 10) to rats, 5 days per week for 9-22 months (*Hirsch and Johansson 1983*). Those rats received 0.2 g ST twice each day, with an estimated daily exposure time of 12 hours. Untreated rats (n = 15) with identical surgical lip canals were used as controls. The snus group exhibited epithelial hyperplasia, including hyperorthokeratosis (hyperkeratosis with a granular cell layer and loss of nuclei in keratin layer) and acanthosis (thickening of the epidermal spinous cell layer). The alkaline snus treatment group experienced similar effects that were accompanied by focally atrophic and ulcerated epithelium and less fibrosis. One rat exposed to snus developed an oral squamous cell carcinoma.

Hirsch again used surgical lip canals in a small (10 rats/group) 18-month chronic study of snus administered to rats infected with HSV-1 (*Hirsch et al. 1984*). Rats exposed to snus alone exhibited primarily benign lesions. Two rats exposed to both HSV-1 and snus developed oral squamous cell carcinomas.

The surgical lip canal model was also used to evaluate the reversibility of snus-induced lesions (*Hirsch et al. 1986*). This phenomenon is observed in human snuff users who discontinue the practice (*see e.g., Wallström et al. 2011*). Thirty female rats were exposed to snus (0.2 g twice daily) for a period of 13 months. The rats were divided into three test groups. The first group was sacrificed immediately following the 13-month exposure period. The snus was removed from the other two test groups at that time, and those groups were terminated at 1 month and 4 months, respectively, after ceasing snus exposure. All rats sacrificed after 13 months displayed benign oral lesions, but such lesions were markedly reduced or absent following the snus-free intervals.

In a two-year surgical lip canal study in rats, [Hecht et al. 1986](#) found a low incidence of papillomas in the oral cavity for groups of Fischer 344 rats treated with moist snuff alone (2/32 animals), with water-extracted moist snuff having reduced TSNA content (2/21), and moist snuff enriched with aqueous snuff extract sufficient to double its TSNA content (1/32), in addition to a single squamous cell carcinoma inside the test canal for the moist snuff-only treatment group. Neither the oral carcinoma nor the extra-oral tumor incidence in the snuff-treated groups were determined to differ statistically from those of the control group. It is noted that a prior review of this study erroneously reported significant increases in “liver tumors” among animals treated with the extract-enriched snuff (6 in treated animals vs. 1 in controls) ([SCENIHR 2008](#)). An examination of the published paper, however, indicates that these liver lesions were in fact hyperplastic nodules rather than tumors.

Johansson and colleagues subsequently assessed the promotion potential of a commercial U.S. snuff in a 2-year study using rat lip canals. The experiment used twice-daily insertions of 100 mg or more of the tested snuff, and a treatment group exposed to snuff alone was compared with another group exposed to snuff following initiation with 4-NQO¹ (0.5%). The doses employed in this study exceeded the MTD, as marked decreases in body weight gains and increases in moribund animals were seen. Although a low but statistically significant increase in overall tumor incidence was reported among rats treated with snuff alone or 4-NQO/snuff, similar overall tumor incidence was observed between the two treatment groups. The statistical significance of the snuff-only group’s tumor counts was attained only by inclusion of nasal tumors arising from cells of a different histogenic origin, a practice contrary to recommended guidelines for rodent carcinogenesis studies ([McConnell et al. 1986](#)). The authors concluded that snuff exhibited no tumor-promoting effect. Lip sarcomas were also reported in the snuff and 4-NQO/snuff groups, though the authors speculated that these tumors may have developed due to a “significant inflammatory reaction” caused by the coarse and irritating properties of the snuff chosen for the study ([Johansson et al. 1989](#)).

[Larsson et al. 1989](#) assessed the initiation and promotion potentials of Swedish snuff after 16-30 months of treatment in rats initiated with 4-NQO or inoculated with HSV-1. No snuff promoting effects were evident following initiation with 4-NQO. Rats treated with both HSV-1 and snuff did show an increase in tumors outside the oral cavity.

A second chronic surgical canal study of 100-104 weeks duration by Johansson’s group investigated the complete carcinogenic and promotion potential of Kentucky 1S3 Research moist snuff following initiation using either 4-NQO (0.5% solution) or DMBA² (0.1% solution) ([Johansson et al. 1991a](#)). Twice-daily insertions of 150-200 mg snuff for the course of the study produced no significant promotion of oral carcinomas, but snuff promotion resulted in 25 lip

¹ 4-Nitroquinoline-1-oxide is a water-soluble carcinogen capable of inducing oral tumors in laboratory rodents without an extensive inflammatory response. 4-NQO forms DNA adducts and induces intracellular oxidative stress, leading to mutations and DNA strand breaks ([Vitale-Cross et al. 2009](#)).

² 7,12-Dimethylbenz[a]anthracene is a polycyclic aromatic hydrocarbon (“PAH”) used extensively as a model carcinogen known to induce tumors in the skin and mammary glands of experimental animals. DMBA is also immunotoxic to the spleen, thymus, and bone marrow ([Miyata et al. 2001](#)).

sarcomas in the group (n=38) initiated with 4-NQO. The observation of 20 total tumors throughout the body, with 11 in the oral cavity, in the 4-NQO initiation-only group suggests that the five-fold higher dose of the initiator used in this study relative to the authors' prior 1989 experiment exceeded the threshold dose of this genotoxic carcinogen. Similarly, 14 total tumors were recorded in the DMBA initiation-only group, compared to 5 in sham-treated controls. DMBA initiation followed by snuff exposure also failed to demonstrate any promotional effect on oral epithelial tumors, but resulted in 9 lip sarcomas vs. 1 in control animals. The dominant tumor types observed in the snuff-only (10 tumors) and snuff promotion groups (10 and 25 tumors) were sarcomas, tumors of mesenchymal as opposed to epithelial origin. This unusual finding may be related to the placement of the tested snuff doses in the surgically-created canals, with consequent mechanical trauma and fibrosis in deep mesenchymal tissues such as connective tissue and muscle (see p. 1023 in [Grasso and Mann 1998](#)), an exposure scenario very unlike that of actual smokeless tobacco usage. The authors concluded that the 4-NQO served as a more potent tumor initiator than DMBA, and further that snuff is a promoter of 4-NQO-initiated oral tumors under the conditions of the study. The occurrence of 2 benign lip papillomas and 3 squamous carcinomas of the palate in the snuff-only group indicates that moist snuff has a weak carcinogenic potential with regard to epithelial tumors in this experimental model.

A third 108-week chronic study by Johansson's group evaluated the promoting ability of a commercial U.S. snuff alone or following initiation with 4-NQO (0.5%) ([Johansson et al. 1991b](#)). Twice-daily treatments, 5 days/week, of snuff alone resulted in modest but statistically significant increases in total counts of benign (2) and malignant (4) oral epithelial tumors, in addition to 2 lip sarcomas. The 4-NQO-initiated group promoted with snuff exhibited one oral carcinoma and 3 lip sarcomas among 29 surviving animals vs. none in the initiation-only or control groups. The authors concluded that snuff and 4-NQO alone are capable of inducing both benign and malignant tumors, but that snuff lacked promoting ability following initiation with 4-NQO.

A fourth subchronic study by Johansson's group evaluated the possible immune effects of snuff administered to 38 adult male Sprague-Dawley rats via surgical lip canals for 15 weeks ([Johansson et al. 1991c](#)). The rats were given 150 mg of snuff twice daily, five days per week. The authors reported a detectable and significant reduction in natural killer (NK) cell numbers in peripheral blood, but, as might be expected for a subchronic study, found no neoplasms.

Rat surgical lip canals were also used to investigate the effects of Swedish snuff and HSV-1 on subepithelial mast cells ([Sand et al. 2002](#)). The amount of countable subepithelial mast cells decreased significantly when the rat oral mucosa was exposed to the potent oral carcinogen 4-NQO, but the effect of snuff/HSV-1 was weak. Overall tumor incidence was low, and too few animals were used for reliable statistical analysis to be conducted.

A 12-month study assessed brand-specific responses to multiple ST products using test groups of 15 rats exposed twice daily via surgical lip canals ([Schwartz et al. 2010](#)). All of the tested

products induced sites of moderate to severe dysplasia, with low-nitrosamine ST producing significantly fewer dysplastic changes. No tumors were observed.

6.1.4.2.2.3 Published oral swab studies

Oral swabbing studies have been reported in both mice ([Park et al. 1985](#); [Park et al. 1987](#)) and rats ([Chen 1989](#); [Hecht et al. 1986](#); [Mendel et al. 1986](#); [Mendel et al. 1987](#)). The technique involves swabbing ST extracts into the oral cavity over a period of weeks and assessing any adverse changes. Technical limitations, such as the short duration of ST extract retention in the oral cavity, limit the relevance of this technique in reflecting human ST use.

A chronic study of moist snuff and tobacco-specific nitrosamine (TSNA) effects reported no tumors in F344 rats swabbed with a snuff extract once or twice daily for 131 weeks ([Hecht et al. 1986](#)). Rats swabbed 1-2 times daily with high levels of NNN³ (68 µg/dose) and NNK⁴ (14 µg/dose) exhibited a statistically significant increase in benign oral papillomas (8/30), but not when the nitrosamine solution was mixed with snuff extract (3/30). The authors speculated that snuff extract may actually contain inhibitors of NNN and NNK metabolic activation. Later work by this group provided evidence in support of this hypothesis with the demonstration that nicotine inhibits the metabolism of both NNN and NNK in the oral tissue of rats ([Murphy and Heiblum 1990](#)).

Two studies investigated the effects of swabbing mice over 2-3 months with snuff extract, both with and without HSV inoculation ([Park et al. 1985](#); [Park et al. 1987](#)). The snuff-only and HSV-only treatment groups showed no effect, while the snuff + HSV treatment groups exhibited hyperplasia (increased cell production in normal tissue) and hyperkeratosis (thickened keratin layer of surface epithelium). In contrast to Park's previous studies of HSV and snuff in hamster buccal pouches, no oral tumors were reported in the mouse model.

An oral swab protocol intended to extend oral retention time was described in two additional studies ([Mendel et al. 1986](#); [Mendel et al. 1987](#)). ST extract was mixed with orabase (a mucosa adhesive) prior to swabbing in rats. Orabase mixed with pumice was used as a control. Studies of 1 and 3 months duration reported pre-keratinization changes and increased mitotic activity, but no tumors. The relatively short duration of the study was likely insufficient for any potential tumor development.

The potential of moist snuff to induce oral cancer was investigated by the weekly insertion of the moistened tobacco from a 0.4 gram pouch of a commercial moist snuff bilaterally in the mandibular mucobuccal folds of 15 male and 15 female Harwell Mouth Tumor (HMT) rats for a period of one year, followed by 6 months of further observation. This outbred, Wistar-derived rat strain has been reported to have increased susceptibility to developing squamous cell carcinomas in the oral cavity. The applied tobacco was likely swallowed gradually after these applications. Suggestions of mitotic disruption were noted, and observed histological changes

³ N'-Nitrosornicotine

⁴ 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone

included hyperkeratosis, acanthosis, and sub-epithelial connective tissue hyalinization in the buccal mucosa. These changes were described as resembling those observed in human snuff users. No tumor formation was observed in the experimental animals (Chen 1989).

6.1.4.2.2.4 Published rodent feeding studies

Studies conducted by subchronic or chronic exposures to smokeless tobacco by dietary supplementation achieve both chronic, local exposure of the oral cavity as well as systemic exposures of other organs to smokeless tobacco and its constituents in a way that avoids invasive surgical manipulations required by the lip canal technique. Oral feeding studies provide the additional advantage of substantial systemic exposure to absorbed smokeless tobacco constituents to provide information on potential toxicity and carcinogenicity in other organs, including those of the respiratory, cardiovascular and digestive systems.

DiPaolo conducted one of the earliest chronic toxicity feeding studies of snuff, examining both male rats and male mice in an 18-month study (DiPaolo 1962). Wistar rats received snuff admixed with feed (1 part snuff: 20 parts feed, by weight), while two strains of mice received snuff at levels of 1 part snuff: 4 parts feed (first month), 1 part snuff: 9 parts feed (next 4 months), and finally 1 part snuff: 18 parts feed for the remaining 13 months of the experiment. The rats and mice exhibited significantly decreased food intake, accompanied by a significant dose-related decrease in body weight and significantly decreased survival. Only 40% of the rats survived the snuff treatments, while 98% of the treated mice survived through study termination. Necropsy of the laboratory animals revealed a few pathological changes in four of 16 surviving rats (one with leukocytic leukemia, one with kidney sarcoma, and two with superficial stomach ulcers). Twenty-three mice displayed nephritis, as opposed to two among control animals receiving the normal diet. Since the animals' maximum tolerated dose was greatly exceeded in this crude, early experiment that resulted in 60% mortality in the snuff treatment group of rats, the finding of four instances of organ histopathology among the animals is not interpretable by contemporary standards for such studies.

Homburger *et al.* fed two carcinogen-susceptible lines of inbred male Syrian hamsters a diet containing 20% moist snuff for 2 years in order to ascertain the carcinogenic potential of snuff alone and in concert with a presumed sub-threshold dose of the known carcinogen 20-methylcholanthrene (Homburger *et al.* 1976). Serum cotinine in the test animals far exceeded levels observed in human smokers, and the authors concluded that 20% snuff in the diet was neither carcinogenic nor co-carcinogenic for the test animals.

Feeding studies of smokeless tobacco extracts (STEs) in rats have reported increases in hepatic mitochondrial and microsomal lipid peroxidation, hepatic DNA damage, and excretion of urinary byproducts of lipid peroxidation (Bagchi *et al.* 1994). Whereas this study did not directly assess the potential carcinogenicity of smokeless tobacco extracts, it demonstrated the sensitivity of rats to liver toxicity induced by STEs at sufficiently high doses. The liver is not generally regarded to be a prominent target organ for tobacco-induced toxicity in humans, and so the findings may have limited relevance to conditions of human smokeless tobacco usage.

Ninety-day feeding studies of a snus tobacco blend and an aqueous extract of that blend were conducted in Wistar rats and CD-1 mice ([Theophilus *et al.* 2012](#)), with control groups receiving normal laboratory diet or diet supplemented with nicotine tartrate at levels appropriate to deliver three levels of nicotine that matched those of the groups receiving the snus tobacco blend and extract. This study is discussed in detail in [Section 6.1.4.3](#) presenting scientific data for RJRT-sponsored studies specific to Camel Snus (studies CN49730E and CN49730F). Comprehensive clinical chemistry, histopathologic, and observational data were collected in these GLP-compliant studies. Findings at study termination in the groups of rats and mice receiving the snus tobacco blend or extract were limited to reductions in feed consumption and body weights, most significantly in the high-dose groups, with similar body weight depression noted in the nicotine positive control animals. The snus blend and extract subchronic treatments produced no gross or microscopic pathologic findings in any organ or tissue. This study found rats to be more sensitive to the body weight changes than mice.

[Theophilus *et al.* 2015](#) recently reported a chronic toxicology and carcinogenicity study of a smokeless (snus) tobacco blend and an extract prepared from that blend that were administered in the diet of male and female Wistar-Han rats at levels intended to deliver systemic nicotine at levels spanning and exceeding those experienced by smokeless tobacco users. The findings of this study are detailed in the [Section 6.1.4.3](#) discussion of the [CN49730G Final Toxicity Report](#) and [CN49730G Final Carcinogenicity Report](#) that present scientific data specific to Camel Snus. Except for reductions in body weights, neither the Camel Snus tobacco blend nor its extract produced any clinically-significant toxicity to any organ or system, and no increases in benign or malignant tumors at any site were attributed to the Camel Snus treatments.

[Yu *et al.* 2016](#) reported a 6-month study in which 20 male and 20 female Sprague-Dawley rats received one of three daily gavage doses of an extract prepared from an unspecified Chinese smokeless tobacco sample. Following the 184-day dosing interval, a subgroup of animals of each sex were maintained for an additional 30-day recovery period without dosing to evaluate the reversibility of any observed toxicologic findings. The low, intermediate and high-dosed groups received smokeless tobacco extracts sufficient to achieve respective exposures of 3.75, 7.50 and 15.00 mg nicotine/kg body weight/day, all of which are greatly in excess of those that would be anticipated for heavy smokeless tobacco users. The authors did not report any biomarker data for nicotine or other constituents of the tested extract, as is the contemporary standard for such studies to document the accuracy of the animal dosing. Comprehensive histopathologic, serum chemistry and hematology data were gathered at necropsy, as well as after the 30-day post-dosing recovery period. Decreases in body weight gains and decreased heart, liver and kidney weights were observed in the intermediate and high dose male groups relative to control animals dosed similarly with distilled water. Female animals showed similar effects in the low-dose group as well. Hematology findings were within normal ranges, whereas serum chemistry findings included dose-related elevations in bilirubin, creatinine, AST and ALT, which are commonly observed following acute liver or kidney injury. Histopathologic findings included keratinized stratified epithelium in the esophagus; instances of inflammation, swelling and degeneration in the liver, stomach and spinal cord; and degeneration, swelling and

inflammatory cell infiltration of the kidney. Foci of foam cells (*i.e.*, fat-laden macrophages) in the lung were recorded in some animals of all of the dosed groups, possibly as a consequence of gavage dosing errors. The observed changes were reversible, as they were found to largely or completely resolve over the course of the 30-day post-dosing recovery period. The authors concluded that their study had demonstrated that the smokeless tobacco extracts had “a moderate and reversible toxic effect on the esophagus, stomach, liver, kidney and lung,” and suggested that these target organs be prioritized in future mechanistic studies (Yu *et al.* 2016).

6.1.4.2.2.5 Published transgenic rodent models

Stenström and colleagues investigated the stomach carcinogenicity of snus alone and in combination with *Helicobacter pylori* (*H. pylori*) (Stenström *et al.* 2007). The global prevalence of *H. pylori*, an IARC Group I carcinogen, is over 50%, with a U.S. prevalence of approximately 32.5% (IARC 2012). Snus mixed with rat chow at a concentration of 5–9% was given to wild-type (WT) and gastrin transgenic (INS-GAS) male mice (FVB/NTac) with or without *H. pylori* infection. These mice secrete abnormally high levels of gastrin, and as a result are highly prone to the development of gastric cancer. The transgenic INS-GAS mice receiving 6 months of snus + *H. pylori* all developed gastric carcinoma. Without *H. pylori* infection, 50% of the INS-GAS mice exposed to snus developed gastric carcinoma. Fifty-three percent of WT mice that were infected with *H. pylori* developed gastric carcinoma after exposure to snus for 6 months. WT mice exposed to snus alone, however, developed only “mild morphological changes.” Methodological shortcomings in this study, including the lack of groups of WT or INS-GAS mice exposed to *H. pylori* alone, make observations regarding the interaction of snus and *H. pylori* impossible.

Song *et al.* 2010 recently investigated the potential carcinogenic effects of tobacco in an animal model of chronic pancreatitis. Elastase-IL-1 β transgenic and WT mice were fed diluted tobacco smoke (“TS”) water or snus-containing diet for up to 15 months, and monitored for phenotypic and molecular pancreatic effects. Both TS water- and snus-treated transgenic mice, but not WT mice, developed significant pancreatic ductal epithelial flattening and severe glandular atrophy compared with untreated transgenic mice. Despite these effects, no more advanced pre-neoplastic or neoplastic pancreatic lesions were detected in the transgenic mice exposed to TS water or snus.

6.1.4.3 *In vivo* studies of the potential toxicity and carcinogenicity of Camel Snus

R.J. Reynolds Tobacco Company has sponsored a series of *in vivo* studies to evaluate the potential systemic toxicity and carcinogenicity of the Camel Snus tobacco blend. These studies included both preliminary investigations to develop information on appropriate doses, as well as subchronic and chronic studies in rats and mice that were performed in accordance with applicable provisions of Good Laboratory Practices guidelines (21 CFR Part 58, as amended 21 May, 2002). These studies are listed in Table 6.1.4-2 below and discussed in the subsequent text.

Table 6.1.4-2: Index of RJRT *in vivo* studies

Reference	Title	Tested Tobacco Blend; Relevance to Camel Snus Styles ^a
TOX209 Final Report	Two Week Investigational Study of the Palatability of Smokeless Tobacco Blend and Extract Formulated in NTP-2000 Diets for Rats	Frost, Frost Large, Mellow, Mint, Robust, Winterchill
TOX210 Final Report	Two Week Investigational Study of the Palatability of Smokeless Tobacco Blend and Extract Formulated in NTP-2000 Diets for Mice	
TOX213	Two Week Repeat Investigational Study of the Palatability of Smokeless Tobacco Blend and Extract Formulated in NTP-2000 Diets for Mice at Higher Doses	
CN49730C	28-Day Repeated Dose Toxicity Study of Tobacco Blend and Aqueous Tobacco Extract in Wistar Han Rats	
CN49730D Final Report	28-Day Repeated Dose Toxicity Study of Tobacco Blend and Aqueous Tobacco Extract in CD-1 Mice	
CN49730E Amended Final Report	90-Day Repeated Dose Subchronic Toxicity Study of Tobacco Blend and Aqueous Tobacco Extract in Wistar Han Rats	
CN49730F Amended Final Report	90-Day Repeated Dose Subchronic Toxicity Study of Tobacco Blend and Aqueous Tobacco Extract in CD-1 Mice	
CN49730G Final Toxicity Report	2-Year Chronic Toxicity/ Carcinogenicity Feeding Study of Tobacco Blend and Aqueous Tobacco Extract in Wistar Han Rats: 12-Month Repeated Dose Chronic Toxicity Study	
CN49730G Final Carcinogenicity Report	2-Year Chronic Toxicity/Carcinogenicity Feeding Study of Tobacco Blend and Aqueous Tobacco Extract in Wistar Han Rats: 2-Year Carcinogenicity Study	

^a All Camel Snus styles are manufactured with an identical tobacco blend, so the findings of this series of *in vivo* studies that investigate that blend and an aqueous extract of that blend are relevant to all six styles (Frost, Frost Large, Mellow, Mint, Robust, Winterchill).

6.1.4.3.1 RJRT 14-day range-finding studies in rodents for Camel Snus

Prior to conducting the 2-year chronic study, RJRT carried out a series of 14-, 28-, and 90-day feeding studies. These studies were performed to ensure appropriate chronic study dosages, to identify potential target organs, and to characterize any subchronic treatment-related effects of ingesting the Camel Snus tobacco blend or an aqueous extract of that blend. The rodent feeding studies were conducted using CFW Swiss Webster/CD-1 mice and Wistar Hannover (Wistar Han) rats.

Three 14-day, non-GLP pilot feeding studies ([TOX209 Final Report](#), [TOX210 Final Report](#), [TOX213](#)) were conducted to find appropriate dose ranges for longer-term studies and to clarify palatability issues surrounding test article administration. The Camel Snus tobacco blend, aqueous extract of the same blend, or nicotine tartrate (positive control) were fed to Wistar Han rats or CFW Swiss Webster mice at dosages ranging from 0.2-40 mg nicotine/kg body weight per day. The mouse study was repeated ([TOX213](#)) at 40-400 mg nicotine/kg body weight per day due to a lack of statistically significant differences in final body weights across the initial dosage range. Aside from a dose-dependent decrease in body weight gain, no treatment-related effects were noted during clinical observations.

6.1.4.3.1.1 [TOX209 Final Report](#) : Two Week Investigational Study of the Palatability of Smokeless Tobacco Blend and Extract Formulated in NTP-2000 Diets for Rats

Purpose

The objective of this non-GLP study was to evaluate the palatability of diets formulated with a smokeless tobacco blend, an aqueous tobacco extract of the tobacco blend, or nicotine tartrate (as a positive control) when fed to Wistar Han rats. The dose range tested was 0.2-40 mg nicotine/kg bw/day.

Design

Palatability was assessed by comparing the body weights of rats fed diets with increasing concentrations of the smokeless tobacco blend, the tobacco extract and the positive control to the body weights of the negative control mice fed the standard feed with no additions. The duration of the feeding and data collection period was 14 days. Feed intake and body weight were measured daily during the study. Mortality and morbidity observations were conducted twice daily. Standard clinical observations were conducted twice weekly.

Results

The effect on body weight produced by feeding diets formulated with the test articles and positive control was clearly seen when expressed as cumulative percent body weight gain. Cumulative percent body weight gain was almost identical between extract and blend groups, with doses of 0.2-40 mg nicotine/kg bw/day demonstrating dose-related trends in body weight gains that were clearly different from those of the diet control group. Rats fed the 20 mg/kg bw/day extract did not statistically differ from the control group at the end of the study. Body weight decreases at 40 mg nicotine/kg bw/day were excessive for both test articles and the positive control, so the high dose was not considered appropriate for use in longer term studies. Body weight gain was more depressed with the positive control than with either of the test articles. At 20 mg nicotine/kg bw/day, the trend was similar to that of the tobacco blend but greater than that seen with the tobacco extract.

6.1.4.3.1.2 TOX210 Final Report: Two Week Investigational Study of the Palatability of Smokeless Tobacco Blend and Extract Formulated in NTP-2000 Diets for Mice

Purpose

The objective of this non-GLP study was to evaluate the palatability of diets formulated with a smokeless tobacco blend, an aqueous tobacco extract of the tobacco blend, or nicotine tartrate (as a positive control) when fed to male, CFW Swiss Webster/CD1 mice. The dose range tested was 0.2-40 mg nicotine/kg bw/day.

Design

Palatability was assessed by comparing body weights of mice fed diets with increasing concentrations of the smokeless tobacco blend, the tobacco extract and the positive control to the body weights of the negative control mice fed the standard feed with no additions. The duration of the feeding and data collection period was 14 days. Feed intake and body weight were measured daily during the study. Mortality and morbidity observations were conducted twice daily. Standard clinical observations were conducted twice weekly.

Results

By the end of the study, there were no statistically significant differences in final body weights at the doses tested (0.2-40 mg nicotine/kg bw/day). Therefore, the major recommendation was to repeat the mouse study using higher doses (starting with 40 mg nicotine/kg bw/day).

6.1.4.3.1.3 TOX213: Two Week Repeat Investigational Study of the Palatability of Smokeless Tobacco Blend and Extract Formulated in NTP-2000 Diets for Mice at Higher Doses

Purpose

The objective of this non-GLP study was to evaluate the palatability of diets formulated with a smokeless tobacco blend, an aqueous tobacco extract of the tobacco blend, or nicotine tartrate (as a positive control) when fed to male, CFW Swiss Webster/CD1 mice. The dose range tested was 40-400⁵ mg nicotine/kg bw/day.

Design

Palatability was assessed by comparing the body weights of mice fed diets with increasing concentrations of the smokeless tobacco blend, the tobacco extract and the positive control to the body weights of the negative control mice fed the standard feed with no additions. The duration of the feeding and data collection period was 14 days. Feed intake and body weight were measured daily during the study. Mortality and morbidity observations were conducted twice daily. Standard clinical observations were conducted twice weekly.

Results

By the end of the study, groups dosed at 40 and 80 or less mg nicotine/kg bw/day showed no statistical difference in body weights compared to untreated controls. Groups dosed at 240 mg nicotine/kg bw/day or greater demonstrated severe dose-dependent reductions in body weight and were returned to Control feed prior to completion of the study. Study data indicated that a dose of 160 mg nicotine/kg bw/day was close to or slightly higher than a Maximum Tolerated Dose (MTD) for male, Swiss Webster mice as defined by a 10% decrease in body weight.

The dose and dose schedule data collected in the preliminary studies discussed briefly above was used in the design of the subsequent subchronic and chronic toxicity and carcinogenicity studies that are summarized below. These studies included the dietary administration of both the Camel Snus tobacco blend or an aqueous extract of that tobacco blend admixed with a standard laboratory animal diet, and were performed in accordance with applicable provisions of Good Laboratory Practices (21 CFR Part 58, as amended 21 May, 2002).

6.1.4.3.2 RJRT 28-day rodent subchronic toxicity studies of Camel Snus

Two 28-day, GLP-compliant feeding studies ([CN49730C Final Report](#), using Wistar Han rats; [CN49730D Final Report](#), using CD-1 mice) compared the short-term toxicity of Camel Snus tobacco blend, an aqueous extract of the same blend, and appropriate controls (nicotine tartrate positive control, diet only negative control). For the mouse study, doses ranged from 2-200 mg nicotine/kg body weight/day and near-complete mortality (unscheduled termination)

⁵ Low dose was chemically confirmed to be approximately 4 mg nicotine/kg bw/day instead of 40 mg nicotine/kg bw/day.

occurred in the highest dose groups. Rat study doses ranged from 0.2-20 mg nicotine/kg body weight/day. Although no treatment-related mortality was observed, exposure to the test articles at 20 mg/kg caused decreases in mean body weight and feed consumption sufficient to preclude selection of that dose for longer-term studies. Organ weight changes were observed in both studies, but no gross lesions were detected at necropsy in any of the evaluated organs.

6.1.4.3.2.1 CN49730C: 28-Day Repeated Dose Toxicity Study of Tobacco Blend and Aqueous Tobacco Extract in Wistar Han Rats

Purpose

The objective of this GLP-compliant study was to compare the short-term toxicity of feeding a tobacco blend, aqueous tobacco extract, nicotine tartrate positive control and diet negative control to rodents. The study also aimed to differentiate any palatability-related effects from toxic effects by including pair-fed groups corresponding to the high dose groups from which food consumption was measured daily. Plasma concentrations of nicotine and cotinine were also determined.

Design

Three hundred eighty-eight male and female rats were randomized into 14 groups. The study consisted of a 28-day toxicity study that included a neurotoxicity component and a toxicokinetic study. Endpoints used to evaluate the potential toxicity of tobacco blend and aqueous tobacco extract were clinical observations, body weights, body weight changes, food consumption, clinical pathology, functional observational battery, gross necropsy, and selected organ weights. Toxicokinetic evaluations of nicotine tartrate, tobacco blend, and aqueous tobacco extract were performed on Days 13, 14, and 28. The doses were 0, 0.2, 2, 8, and 20 mg nicotine/kg bw/day. 10 animals/gender/group were included in the main study, with 6 animals/gender/group used for toxicokinetic studies. Control diet pair-fed groups were included for those groups receiving the highest dosage of the test articles.

Results

- No treatment-related mortality occurred in any of the core test groups indicating that the dosages were appropriate for the dosed feed route of administration.
- Exposure of rats to blend and extract at 8 and 20 mg/kg bw/day caused a dose-related reduction in group mean absolute body weight and food consumption relative to the respective control groups. Exposure to the test articles at 20 mg/kg bw/day caused decreases in both group mean body weight and food consumption which would preclude selection of this dose for the 90-day study. Moreover, the magnitude of the reduction in the high dose group for each test article was generally greater than that of their respective pair-fed control group. Exposure to the test articles at 8 mg/kg bw/day caused more moderate reductions in group mean body weight and food consumption relative to control.

- Toxicokinetic studies showed that plasma nicotine and cotinine values increased proportionally with an increase in dose for the blend and extract groups.
- No clinical signs of toxicity were observed during this study that could be directly attributed to the administration of any of the test or control articles. Treated animals were generally similar to control in overt behavior and in general health and appearance.
- In the functional observational battery, the 20 mg nicotine/kg bw/day test groups revealed subtle changes in arousal and rectal temperature. For example, high dose female rats in the nicotine tartrate, blend, and extract treatment groups showed a reduction in mean rectal temperature as compared to the female control group. The neurobehavioral effects appeared to be directly related to the nicotine component of each of the test articles since these effects were apparent with both test articles and the positive control.
- Trends in clinical chemistry occurred in groups given the test articles at the highest dose and the positive control and included: decreased total protein, albumin, and globulin and decreased glucose with increased alkaline phosphatase, total and direct bilirubin, blood urea nitrogen, albumin/globulin ratio, triglycerides, and cholesterol. The alterations in these parameters were of similar magnitude in both sexes. Comparison of results with pair-fed controls indicated that the alterations were not simply due to decreased food consumption and were more likely associated with exposure.
- Changes in absolute organ weight and organ to body and/or brain weight values were attributed to treatment-related reductions in group mean body weight, since no gross lesions were detected at necropsy in any of the affected organs.

6.1.4.3.2.2 CN49730D Final Report: 28-Day Repeated Dose Toxicity Study of Tobacco Blend and Aqueous Tobacco Extract in CD-1 Mice

Purpose

The objective of this GLP-compliant study was to evaluate the short-term toxicity of a tobacco blend and aqueous tobacco extract in comparison to the nicotine tartrate positive control and diet negative control in CD-1 mice.

Design

Five hundred ninety-eight male and female mice were randomized into 10 dose groups. The 28-day toxicity study included both a neurotoxicity component and a toxicokinetic component. Endpoints used to evaluate the potential toxicity of tobacco blend and aqueous tobacco extract were clinical observations, body weights, body weight changes, food consumption, clinical pathology, functional observational battery, and gross necropsy including selected organ weights. Toxicokinetic evaluations of plasma nicotine and cotinine were performed on Days 14, 15, and 28. The doses were 0, 2, 20, 80, and 200 mg nicotine/kg bw/day. Ten

animals/sex/group were included in the core study; 5 (control) or 23 (experimental) animals/sex/group were included in the toxicokinetics study.

Results

- In the mouse core study, unscheduled terminations occurred in high dose groups (entire B200M, B200F, E200M; 6/10 E200F and 4/10 NT200M).
- The high dose (200 mg nicotine/kg bw/day) groups exhibited a decrease in food consumption, a decrease in body weight gain, and showed clinical signs of toxicity indicative of inanition and stress, indicating these doses would be too high for a 90-day study due to a lack of palatability of the dosed feed. Survival decreased in mice given the highest dose groups. At high doses, male mice generally appeared more susceptible than female mice at comparable dose levels.
- The 200 mg nicotine/kg bw/day dose groups had depressions in group mean body weights >17% (vs. Control). B80M and E80M showed ~10% depressions in group mean body weights relative to Control with decreases in food consumption of 16.7 and 8.3%, respectively. The B80F and E80F groups showed group mean body weights that were -1.2% and +0.8% of the CF group value. Food consumption values of the B80F and E80F were depressed 18.2 and 11.4%, respectively, relative to that of the CF group.
- The functional observational testing revealed subtle changes in arousal and rectal temperature. For example, the E80M dose group showed increased home cage arousal (similar to the NT200M group); more arousal and less sleeping (than the B80M); and decrease in rectal temperature (significantly lower than that of the CM group). Female mice also showed increased resistance to removal from the home cage when compared to the CF group. The NT200M group showed a reduction in mean rectal temperature relative to the CM group, and the mean rectal temperatures of the B80M, E20M, and E80M groups were numerically lower than the CM group.
- Clinical pathology findings (NT200M, E80M, and E200F) revealed subtle changes including decreased hemoglobin and decreased total white cell counts and lymphocyte counts which were similar in magnitude in all affected groups and attributed to stress. Changes in clinical chemistry parameters were sporadic and of little biological significance. Changes in absolute organ weights and in the organ to body weight and brain weight values were attributed to decreases in body weights rather than to any specific direct organ toxicity due the absence of findings at necropsy.
- Dose-concordance was established for the toxicokinetics studies (increased doses resulting in increased plasma nicotine and cotinine).

6.1.4.3.3 RJRT 90-day subchronic rodent toxicity studies of Camel Snus

The subchronic toxicity of the Camel Snus tobacco blend was further assessed in 90-day repeated dose studies in male (M) and female (F) rats ([CN49730E Amended Final Report](#)) and mice ([CN49730F Amended Final Report](#)). Wistar Hannover rats were dosed with the blend (B) and extract (E), based on the results of the 28-day study, at 0.3-6.0 mg nicotine/kg body weight/day. CD-1 mice were dosed at 6-120 mg nicotine/kg body weight/day. Similar dosing with the nicotine tartrate (NT) positive control was performed. Necropsy did not reveal any treatment-related adverse effects on target organs. A few microscopic changes were observed, but all such changes were typical of background lesions and interpreted as not treatment-related. Organ weight changes were attributed to reductions in body weight gain because microscopic examination of organs at necropsy did not reveal any changes that were attributed to exposure. This study, which spanned the NOAEL, LOAEL and MTD for the Camel Snus blend and extract, was the subject of a peer-reviewed publication ([Theophilus et al. 2012](#)) that is included among the technical reports and publications provided in Section 7 of this Application.

6.1.4.3.3.1 [CN49730E Amended Final Report: 90-Day Repeated Dose Subchronic Toxicity Study of Tobacco Blend and Aqueous Tobacco Extract in Wistar Han Rats](#)

Purpose

The objective of this GLP-compliant study was to evaluate subchronic toxicity of a tobacco blend and aqueous tobacco blend extract in comparison to a nicotine tartrate positive control and a normal diet negative control in Wistar Han rats.

Design

Four hundred twenty-six male and female rats were randomized into eight dose groups. The study consisted of a 90-day toxicity study and a toxicokinetic study. Endpoints used to evaluate the potential toxicity of tobacco blend and aqueous tobacco extract were clinical observations, body weights, body weight changes, food consumption, ophthalmic exams, clinical pathology, gross necropsy, selected organ weights, and microscopic exams. Toxicokinetic evaluations of nicotine tartrate, tobacco blend, and aqueous tobacco extract groups were performed at Weeks 2, 4, 8, and 13. Based on the results of the 28-day study, the doses selected for this study were 0.3, 3, and 6 mg nicotine/kg bw/day.

Results

- No treatment related mortality or clinical signs of toxicity occurred over the course of this study. Treated animals were similar to Control in overt behavior and in general health and appearance.
- Plasma nicotine and cotinine data collected at weeks 4, 9 and 13 showed a good dose-response relationship. These levels spanned typical plasma levels for nicotine reported for

smokeless tobacco users, ~30 ng/ml (LSRO 2008; Benowitz 1992) in the lower and middle doses, and substantially exceeded that range in the high-dose group. There were treatment-related changes in group mean body weight in the NT, B, and E groups at the higher levels of exposure. The NT6M, B6M, and E6M dosage groups showed reductions in group mean body weight of 12.7, 13.7, and 9.2% relative to that of their respective control group. The corresponding NT6F, B6F, and E6F dosage groups showed reductions of 11.9, 11.0, and 11.3% relative to that of their respective control group. The reduction in body weight gain generally correlated with reduced food consumption relative to Control in the NT6M, B6M, NT6F, B6F, and E6F dosage groups.

- In spite of the small exposure related reductions in food consumption, the plasma nicotine and cotinine increased approximately proportionally with an increase in the exposure level for both the B and E dosage groups. The NT6, B6, and E6 male and female dosage groups exhibited similar nicotine and cotinine concentrations. Based on toxicokinetic data and body weight changes, male rats were more sensitive than female mice to the effects of NT, B, and E as plasma nicotine/cotinine and percentage body weight reductions were generally lower for the female treatment groups when compared to their corresponding male treatment groups.
- Clinical pathology evaluation included hematology, clinical chemistry, coagulation tests (prothrombin time), and urinalysis and did not indicate any treatment-related findings or trends. Ophthalmic studies conducted at the end of the study did not reveal any treatment related eye abnormalities.
- Necropsy did not reveal any treatment-related target organs. Organ weight changes were not associated with any microscopic findings and were secondary to treatment-related reductions in body weight gain. Microscopic examination did not reveal any changes that were attributed to exposure to NT, B, or E.

6.1.4.3.3.2 CN49730F Amended Final Report: 90-Day Repeated Dose Subchronic Toxicity Study of Tobacco Blend and Aqueous Tobacco Extract in CD-1 Mice

Purpose

The objective of this GLP-compliant study was to evaluate the subchronic toxicity of a tobacco blend and aqueous tobacco blend extract in comparison to a nicotine tartrate positive control and a normal diet negative control in CD-1 mice.

Design

Four hundred ninety male and female mice were randomized into eight dose groups and one group of sentinels. The study consisted of a 90-day toxicity study and a toxicokinetic study. Endpoints used to evaluate the potential toxicity of tobacco blend and aqueous tobacco extract were clinical observations, body weights, body weight changes, food consumption, ophthalmic

exams, clinical pathology, gross necropsy, selected organ weights, and microscopic examination of tissues from Control and high dose groups. Toxicokinetic evaluations of nicotine tartrate, tobacco blend, and aqueous tobacco extract groups were performed at Weeks 3, 5, 9, and 14. Based on the results of the 28-day study, the doses selected for this study were 6, 60, 120 mg nicotine/kg bw/day.

Results

- No treatment related mortality or clinical signs of toxicity occurred over the course of this study. Treated animals were similar to control in overt behavior and in general health and appearance.
- There were treatment-related changes in group mean body weight in the NT, B, and E groups at the higher levels of exposure. The NT120M, B60M, B120M, E60M, and E120M dosage groups showed reductions in group mean body weight of 13.3, 7.1, 13.8, 7.1, and 14.5% relative to that of their respective control group. The NT120F, B60F, B120F, and E120F dosage groups showed reductions of 10.1, 7.4, 6.1, and 10.1% relative to that of their respective control group. The reduction in body weight gain generally correlated with reduced food consumption in these dosage groups.
- In spite of the small exposure-related reductions in food consumption, the plasma nicotine and cotinine values increased accordingly, with an increase in the exposure level for both the B and E dosage groups. Toxicokinetic studies showed a sex effect, as both nicotine and cotinine concentrations were consistently lower in the female dosage groups compared to the male dosage groups.
- Clinical pathology evaluation included hematology, clinical chemistry, and urinalysis and did not indicate any treatment-related findings or trends. Ophthalmic studies conducted at the end of the study did not reveal any treatment-related eye abnormalities.
- Necropsy did not reveal any treatment-related target organs. Organ weight changes were not associated with any microscopic findings and were secondary to treatment-related reductions in body weight gain. Microscopic examination did not reveal any changes that were attributed to exposure to NT, B, or E.

6.1.4.3.4 RJRT chronic toxicity/carcinogenicity study of Camel Snus

Following completion of the subchronic studies, RJRT sponsored a 2-year chronic carcinogenicity/1-year chronic toxicity feeding study ([CN49730G Final Toxicity Report](#) and [CN49730G Final Carcinogenicity Report](#)) of the Camel Snus tobacco blend and an aqueous extract of that blend in Wistar Hannover rats, a species and strain selected for its responsiveness in preliminary studies. The aqueous tobacco extract treatment was intended to simulate the manner of exposure experienced by smokeless tobacco users who hold the product in the mouth rather than swallowing it. Target doses of 0.2, 2.0 and 5.0 mg nicotine/kg body weight/day were chosen to span and exceed the range of nicotine exposures typical for

smokeless tobacco users, and the addition of the tobacco blend and extract were adjusted to maintain dosing at the target levels over the course of the study. No treatment-related clinical signs of toxicity were apparent following the 12-month chronic toxicology component of the study. The treated animals were similar to controls in behavior, health and appearance. Elevation in serum cholesterol for females administered blend or extract at the highest dose of 5 mg nicotine/kg body weight/day was interpreted to be due to test article administration, and is consistent with findings reported in the available literature ([Abd El Mohsen et al. 1997](#)). No treatment related gross lesions were identified at necropsy. Microscopic examination did not reveal any potential target organs at the 12-month interim termination. A few macroscopic and microscopic lesions (non-neoplastic and neoplastic) were observed, and all such findings were typical of spontaneous background changes observed in untreated laboratory rats of this strain ([Mitsumori et al. 2001](#); [Son et al. 2010](#)). These findings were interpreted as incidental to the Camel Snus treatments, and to be neither toxicologically nor biologically significant. The tobacco blend and extract test articles induced similar responses at comparable nicotine-equivalent doses.

Other animal groups were continued on the treatments with Camel Snus tobacco blend and its extract for a second year. Following completion of the 2-year chronic carcinogenicity portion of the study, two tumor types were observed at statistically significant levels vs. controls. These observations were exclusive to the highest tobacco blend treatment groups, and included female uterine carcinoma (7/59) and male epididymal mesothelioma (3/60). Statistically significant decreased incidence relative to control animals was observed for three other tumor types: female mammary gland adenomas, female skin basal cell carcinomas, and male thyroid follicular cell adenomas. All of these tumor findings, typical of spontaneous background occurrences in untreated Wistar Han rats, were interpreted to be neither toxicologically nor biologically significant, and unrelated to the Camel Snus dosing.

The methods and findings of these concurrent chronic toxicity and carcinogenicity bioassays are further detailed below:

6.1.4.3.4.1 **CN49730G Final Toxicity Report: 2-Year Chronic Toxicity/Carcinogenicity Feeding Study of Tobacco Blend and Aqueous Tobacco Extract in Wistar Han Rats: 12-Month Repeated Dose Chronic Toxicity Study**

6.1.4.3.4.2 **CN49730G Final Carcinogenicity Report: 2-Year Chronic Toxicity/Carcinogenicity Feeding Study of Tobacco Blend and Aqueous Tobacco Extract in Wistar Han Rats: 2-Year Carcinogenicity Study**

Purpose

The objective of this GLP-compliant study was to evaluate the toxicity of the Camel Snus tobacco blend (B) or an aqueous extract of the Camel Snus tobacco blend (E), administered in the diet of male (M) and female (F) rats for a period of one year, and to compare any findings to those of an appropriate control (C) group of animals maintained on the normal laboratory diet.

The separate report on the carcinogenicity phase of study ([CN49730G Final Carcinogenicity Report](#)) reported findings for groups of male and female rats that continued with the Camel Snus blend and extract dosing regimen for another year, with evaluation of tumor responses following the 2-year dosing period.

All aspects of the studies were conducted in accordance with applicable provisions of Good Laboratory Practices.

Design

Wistar Hannover (Han) rats were randomized into nine groups of 40 rats each: Five treatment groups; two control groups; and one sentinel group. The treatment groups included low, intermediate and high doses of the Camel Snus tobacco blend (0.2, 2 and 5 mg nicotine/kg bw/day, respectively). Other groups received analogous low, intermediate and high doses of an aqueous extract of the Camel Snus tobacco blend at three doses matched in nicotine content to those of the tobacco blend groups. The dosages were determined by evaluating data on dietary palatability and food consumption gathered in the previous subchronic studies. A comprehensive toxicologic evaluation performed at study termination included clinical observations, body weights, food consumption, clinical pathology, gross necropsy, selected organ weights, and microscopic histopathologic examination.

Results

Survival: No treatment-related mortality occurred in the 12-month toxicology phase that could be definitively attributed to treatment. There were also no treatment-related effects on survival during the 2-year carcinogenicity phase of the study.

Clinical Observations: No treatment-related clinical signs of toxicity were apparent over the course of the study. Treated animals were similar to controls in overt behavior and general health.

Body Weights: Following the 1-year toxicology phase, a significant 9% reduction in group mean body weight relative to control animals was observed for the high dose, 5BM male treatment group receiving 5 mg nicotine/kg/day of Camel Snus tobacco blend. A 15% reduction was observed for the E5F female treatment group receiving the high dose of 5 mg nicotine/kg/day of Camel Snus tobacco blend extract. Females receiving the intermediate dose of 2 mg nicotine/kg/day of extract also exhibited statistically significant weight reduction, but did not exceed 10% difference from their respective controls. Following the 2-year carcinogenicity phase, significant body weight reductions were observed for the B2M and E2M groups (13% compared with CM). For females, the B2F and E2F groups were 17% and 15% reduced compared with CM, while the B5F and E5F groups were 28% and 26% reduced, respectively, compared with CF. Body weights were generally similar between B and E groups at comparable doses.

Toxicokinetics: Based on plasma nicotine and cotinine measurements throughout the 2-year carcinogenicity phase, exposure was appropriate to span and exceed the typical plasma levels for nicotine reported for smokeless tobacco users, ~30 ng/ml (LSRO 2008; see Chapter 4 in Benowitz 1992).

Gross pathology/histopathology: There were no gross or histological findings at the 1-year toxicity or 2-year carcinogenicity phases that would be considered treatment related in the context of available historical data for Wistar Han rats.

Necropsy: Non-neoplastic and neoplastic findings in rats from various groups of both sexes, including controls, were observed following both the 1-year toxicity and 2-year carcinogenicity phases. All findings were typical of spontaneous changes and were consistent with background changes previously reported in untreated Wistar Han rats (Mitsumori *et al.* 2001; Son *et al.* 2010; Charles River 2011).

Tumor Analysis: Following the 2-year carcinogenicity phase, 90 tumor types were identified during the microscopic examination of both sexes. Only two tumor types exhibited statistically significant increasing dose-response trends. These were malignant carcinomas of the uterus in B females, and malignant mesothelioma of the epididymis in B males. Incidence rates for both of these tumor were within the historical ranges expected for untreated Wistar Han rats, and they were deemed to be spontaneous occurrences. There were no statistically significant increasing dose-response trends in any of the extract (E) groups. Statistically significant decreasing dose-response trends were observed for benign mammary gland adenomas (B females), malignant skin basal cell carcinomas (E females) and benign thyroid follicular cell adenomas (E males). The occurrence of spontaneous tumors in aging rats of this strain is discussed further in the published report for this study (Theophilus *et al.* 2015). All of these tumor findings were judged to be unrelated to the Camel Snus blend and extract treatments.

6.1.4.4 Conclusions

6.1.4.4.1 Smokeless tobacco exhibits lower carcinogenicity, and lower respiratory and systemic toxicity in laboratory animals when compared with cigarette smoke

Cigarette smoke condensates have been shown in over 50 years of cumulative research to be unequivocally carcinogenic when applied subchronically or chronically to the dermal epithelium of laboratory mice. These studies produce significantly-elevated basal cell and squamous cell carcinomas in a dose-responsive manner, with increasing tumor incidence and multiplicity and shorter tumor latency with increasing applied doses. Whereas cigarette smoke inhalation studies in animals have not proven to be reliable models of the development of tumors of the respiratory epithelium that resemble those that are elevated among human smokers, they do produce changes in biomarkers of oxidative stress and inflammation, inflammatory cell influx, losses of normal ciliated cells, significant histopathologic changes, and structural damage to the architecture of the lung that resemble analogous changes observed in human smokers. These

changes are believed to underlie the development of smoking-related cardiovascular and respiratory diseases.

In contrast to studies of cigarette smoke and smoke condensates, the published *in vivo* studies of ST provide no consistent evidence of oral carcinogenicity or significant, irreversible systemic or target organ toxicity in studies of up to two years in duration, which approximates the lifetime of laboratory rats. Published studies to date have included a variety of surgical and other techniques to achieve subchronic or chronic topical exposures of the oral cavity of experimental animals to smokeless tobacco, as well as chronic feeding studies to achieve both digestive tract and systemic exposure. Although some oral tumors have been reported in studies using the rat surgical lip canal model, the low numbers of tumors described were plausibly attributed at least partially to mechanical trauma and prolonged inflammation attending this invasive surgical exposure technique. A 1998 comprehensive review of the evidence from this and other animal models regarding ST and oral cancer concluded that the sum total of experimental work in animal systems suggests that ST is not carcinogenic to the oral mucosa of hamsters or rats (Grasso and Mann 1998). Oral feeding and gavage studies have likewise reported smokeless tobacco to have low systemic toxicity and no carcinogenic potential. Overall, these data indicate that the *in vivo* toxicity and carcinogenicity of smokeless tobacco, as it has been investigated to date, are significantly lower than those of cigarette smoke or cigarette smoke condensate when evaluated in subchronic and chronic studies of similar duration.

The *in vivo* animal studies of Camel Snus that were sponsored by RJRT and presented and discussed in detail in this Application were all conducted according to current, well-accepted guidelines governing research with laboratory animals. With the exception of several pilot dose range-finding studies, all of the subchronic and chronic toxicity and carcinogenicity studies were conducted by independent extramural testing facilities in accordance with contemporary good laboratory practices provisions. The findings from these studies concur with prior published evidence for other, broadly similar smokeless tobacco products in demonstrating that Camel Snus has only a minimal toxicologic potential, and no carcinogenic potential in chronic bioassays conducted by oral feeding that expose the oral cavity and other tissues to high levels of the Camel Snus tobacco blend and its constituents. The additional testing of an aqueous extract of Camel Snus was intended to provide a dosing strategy that resembles some aspects of the exposures that occur during smokeless tobacco usage, *i.e.*, an internal exposure to an aqueous extract with expulsion of the residual tobacco material after use.

Whereas animal data in isolation generally cannot be extrapolated directly into the quantification of human risks such as those arising from tobacco use (Stratton *et al.* 2001; Gori 2013; Knight *et al.* 2006), *in vivo* studies considered together with findings from chemical analyses, *in vitro* laboratory testing, and human clinical investigations contribute to a coherent and consistent body of scientific evidence that concurs very well with the findings of epidemiologic studies of smokeless tobacco-using and cigarette-smoking populations in the United States.

6.1.4.4.2 *In vivo* data are consistent with human epidemiology studies

Studies in rodents, along with studies in human populations (epidemiology studies), are the best means currently available for identifying potential human hazards (Rall 2000). For both cigarette smoking and ST, the *in vivo* data are concordant with their respective, markedly different and extensively studied human health risks. Data from both U.S. and Swedish epidemiology studies (Lee 2011; Lee 2013; USDHHS 2014) show that while cigarette smoke is associated with elevated risk for a broad spectrum of adverse effects, the use of ST has little or no adverse effect on the incidence of smoking-related detriments to health, including neoplastic, respiratory and cardiovascular diseases. The largely negative results of the *in vivo* studies conducted on U.S. smokeless tobacco, Swedish snus, and Camel Snus are consistent with epidemiology studies of both historical U.S. smokeless tobacco and Swedish snus that have consistently shown that users of smokeless tobacco experience substantially lower risks for lung cancer, oral cancer and serious respiratory diseases than do cigarette smokers.

This conclusion with regard to *in vivo* testing of U.S. smokeless tobacco in general, and Camel Snus in particular, concurs closely with the findings of lower genotoxicity and cytotoxicity of Camel Snus relative to cigarette smoke in the *in vitro* toxicology testing presented in Section 6.1.3.4 herein, and is likewise very consistent with the smokeless tobacco epidemiology data from both U.S. and other populations that is discussed elsewhere in this Application. Taken together, these independent and complementary lines of scientific evidence provide a sound basis to conclude that smokers who switch completely to exclusive use of Camel Snus will experience reduced risks for lung and oral cancers, as well as other serious diseases associated with smoking.