Section 6.1.2 – Pre-clinical Safety Toxicology

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6.1.2.1 Introduction

The pre-clinical assessment strategy of the Tobacco Heating System (THS) includes a systematic evaluation of biological activity of the produced aerosol *in vitro* and *in vivo* in order to assess its toxicity and its potential for reduced toxicity compared with smoke from cigarettes (CC). One of the most important scientific aspects of preclinically demonstrating the modified risk character of our THS 2.2 is to select the relevant assays to ensure appropriate toxicological testing." For the selection of appropriate tests, scientific literature, scientific knowledge and expertise in this field, as well as published recommendations from different regulatory authorities (U.S. Consumer Product Safety Commission, 1993; International Agency for Research on Cancer, 1986; International Agency for Research on Cancer 1999, U.S. Food and Drug Adminstration, 2000; Centers for Disease Control and Prevention, 2012), have been considered. The selection process was also influenced by relevant and widely accepted U.S. and international guidelines used for defining the testing requirements for regulatory approval of new consumer products, pharmaceutical and chemical agents (e.g. International Conference on Harmonisation, 1995; ICH, 1997; ICH, 2010; OECD, 1997; OECD, 2009a; OECD, 2009b; OECD, 2016).

For the selection of appropriate tests, the following criteria were considered:

1. The test has to be sensitive and responsive to the aerosol generated from THS (representing the test item) and/or CC (representing the reference items).
2. Those tests that are responsive also have to deliver quantitative results and provide possibilities to discriminate between different products (i.e., sufficiently sensitive to assess the actual differences between use of THS 2.2 and use of another tobacco product, although some limitations may apply related to the nature of the assay).
3. The test used should be supported by analytical and biological validation.
4. Its mode of action needs to be sufficiently understood to allow for the biological interpretation of the results.
5. It is important to be able to measure endpoints that may be related to the health effects for which smoking is a risk factor or that are relevant for smoking-related diseases.
6. It is preferable that the tests are representative of, or surrogate for, relevant target organs or tissues.
7. It is important that the tests are accepted by the scientific community or recommended by regulatory and scientific bodies for this or similar purposes.

Based on these criteria, considerations, recommendations, and toxicological knowledge, a battery of *in vitro* assays was selected and performed to assess the cytotoxicity (Neutral Red Uptake Assay) and the genotoxicity of the THS-aerosol. *In vitro* genotoxic assays have been performed in both bacterial (Bacterial mutagenicity assay or Ames Assay) and mammalian cell (Mammalian cell mutagenicity, Mouse Lymphoma Assay, MLA)

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systems. This combination reduces the likelihood of obtaining false negative or false positive results linked to the intrinsic limitations of the single methods (e.g. top concentration of the test substances, nature of the test system, etc.). The aerosol assessed was generated by actively puffing the THS. For cigarette smoke, the smoke tested was mainstream smoke, generated through active puffing of the burning cigarette; the sidestream smoke produced by the cigarette while smouldering was not assessed. For pre-clinical *in vitro* testing the THS-aerosol was separated into two physical fractions comprising either particulate or gas-vapor phase (GVP) constituents. The term total particulate matter (TPM) refers to the particulate phase of the aerosol collected on a Cambridge filter pad, i.e. a glass fiber filter retaining at least 99.9% of all particles having a diameter equal to or greater than 0.3 micrometer of a dioctyl phthalate aerosol at a linear air velocity of 140 $mm.s^{-1}$. GVP is defined as the aerosol/smoke phase that passes through a Cambridge filter pad or cold trap. This aerosol phase is collected by bubbling it through phosphate-buffered saline (PBS) solution. Collection of aerosol subfractions has been used extensively to assess the combined toxicity of their individual constituents when added to the culture medium in which the cells used for the assessment are grown. The subchronic toxicity of the THS-aerosol *in vivo* was evaluated in 90-day inhalation studies in rats. Unlike for *in vitro* testing describe above, in both the animal and several systems toxicology studies (section 7.5.2 of 7.5 Summary), the THS-aerosol was not subfractioned but instead used to directly expose animals or organotypic tissue cultures. Indeed, several *in vitro* systems toxicology studies were conducted using organotypic tissue cultures of airway epithelia grown at the air-liquid interface, allowing the cultures to be exposed to the whole aerosol in a way that closely mimics *in vivo* exposure conditions. All safety toxicology studies (*in vitro* and *in vivo*) were performed in compliance with the OECD Principles of Good Laboratory Practice (OECD, 1998). Results of *in vitro* studies were generally reported on a per mg nicotine basis or when appropriate on a per mg TPM or per item basis, whereas results of *in vivo* studies were reported based on the test atmosphere concentrations of nicotine. There are strengths and limitations to whichever reporting method is chosen, however, reporting results on a per nicotine basis probably represents the most relevant method to the human situation. The rationale being that people adjust the way that they smoke cigarettes to titrate blood nicotine within reasonable limits and therefore expressing results on a per nicotine basis provides a means to compare exposure between different products (Johnson et al., 2009).

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6.1.2.2 *In vitro* studies

Table 1 shows an overview of the *in vitro* studies conducted in the framework of the pre-clinical assessment.

Table 1 Pre-clinical *in vitro* safety toxicology studies

Assay	Protocol	Tobacco Sticks tested	Study report number
Neutral Red Uptake assay (NRU)	INVITTOX 3a	THS Regular: (b) (4) DORADO II / (b) (4) (b) (4) I THS Menthol: (b) (4) /DORADO (b) (4)	RLS-ZRH-2015-250 RLS-ZRH-2015-249
Ames assay	OECD 471	THS Regular: (b) (4) DORADO II / (b) (4) (b) (4) THS Menthol: (b) (4) DORADO (b) (4)	RLS-ZRH-2015-253 RLS-ZRH-2015-254
Mouse Lymphoma Assay (MLA)	OECD 490	THS Regular: (b) (4) (b) (4) THS Menthol: (b) (4) /DORADO (b) (4)	RLS-ZRH-2015-252 RLS-ZRH-2015-251

6.1.2.2.1 Neutral Red Uptake Assay

6.1.2.2.1.1 Introduction

Tobacco smoke and extracts of smokeless tobacco products (e.g SNUS) have been reported in literature to be cytotoxic. Cytotoxicity to tissues may lead to morphological changes and inflammation, depending on the concentration and the type of epithelium. It may contribute to epigenetic effects involved in the promotion phase of the carcinogenic process, which is considered to be very relevant for smoking-related carcinogenesis (Milara and Cortijo, 2012). Moreover, cytotoxicity has also been associated with tissue destruction in smoking-induced pulmonary emphysema (Dey et al, 2010) and endothelial dysfunction as an early step in cardiovascular disease (Tesfamariam and DeFelice, 2007; Siasos et al, 2014). Due to its importance, the cytotoxicity assay has been included in

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most testing batteries for the evaluation of tobacco products; academic and government researchers have considered a cytotoxicity assay to be an important part of a battery of *in vitro* assays recommended for use on tobacco products (Andreoli et al., 2003; Yauk et al., 2012; Thorne et al., 2014; Putnam et al., 2002). The Neutral Red Uptake (NRU) cytotoxicity assay has long been used for the assessment of cytotoxic activity and potency of tobacco smoke and smokeless tobacco products because of its accuracy, reproducibility (Oldham et al., 2012; CORESTA *In vitro* Toxicology Task Force, 2007) and discriminatory power (Roemer et al., 2014; Roemer et al., 2004; Roemer et al., 2008; Zenzen et al., 2012; Richter et al., 2010). This has largely contributed to its wide acceptance and today NRU represents one of the few examples of biological assays, for which a statutory requirement exists (Health Canada, 2004). Indeed, this assay is used for the annual toxicity testing of cigarette brands manufactured in or imported for sale into Canada.

Because of its characteristics, the NRU cytotoxicity assay is considered a useful component of a tiered battery-based assessment of THS aerosol in a weight-of-evidence evaluation of potential for risk reduction.

6.1.2.2.1.2 Methods

The NRU assay was performed in accordance to the INVITTOX protocol 3a (INVITTOX, 1990; Health Canada, 2004). The study quality standards followed the OECD GLP principles. The assay is based upon the uptake of the supra-vital dye Neutral Red by mouse embryo Balb/c 3T3 cells where it accumulates in the lysosomes/endosomes of living cells, while it does not in dead/dying cells. Exposure was performed by incubating BALB/c 3T3 cells for 23 hours +/- 1 hour with TPM or GVP. Three independent batches of TPM and GVP fractions from the test (THS) and reference (3R4F) items were tested in the NRU assay. Three independent NRU tests, meeting the assay acceptance criteria, were required for each item and for each fraction.

At the end of the exposure, the culture medium containing the aerosol fractions was replaced by medium containing the vital dye Neutral Red. After a 3 hour incubation period, the Neutral Red, which was taken up only by viable cells, was determined photometrically from cell lysates. The concentrations that reduced the number of viable cells by 50% (EC50) were determined for the TPM and GVP from THS. Cytotoxicity of the test item aerosol fractions was compared with that from the reference item smoke fractions.

6.1.2.2.1.3 Results and Conclusion

The *in vitro* cytotoxicity of TPM and GVP of the aerosol from the THS regular (THSR) (study RLS-ZRH-2015-250) and THS menthol (THSM) (study RLS-ZRH-2015-249) tobacco sticks and the smoke from 3R4F research cigarette were assessed in the NRU assay using mouse embryo BALB/c 3T3 cells.

A clear dose-dependent decrease in cell viability for both the aerosol fractions generated from the test and reference items was observed. Furthermore, the concentrations used in the tests achieved a range spanning from the maximum to no or little cytotoxicity, allowing an accurate determination of the EC50 and consequently a direct comparison of

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the cytotoxicity of the test and reference item aerosol fractions. Cytotoxicity was determined on a per test item basis, and using the nicotine concentration in the TPM fraction, the cytotoxicity on a per mg nicotine basis was calculated.

On a per mg nicotine basis, the TPM and GVP *in vitro* cytotoxicity of the THSR was reduced by approximately 90% (91.7 % for TPM and 90.2 % for GVP) compared with the 3R4F reference item (Figure 1, Table 2).

Similarly, on a per mg nicotine basis, the TPM and GVP *in vitro* cytotoxicity of the THSM was reduced by approximately 90% (91.8 % for TPM and 90.6 % for GVP) compared with the 3R4F reference item (Figure 1, Table 2).

Based on these results it can be concluded that under the conditions tested and irrespective of the presence of menthol, the cytotoxic activity of TPM and GVP aerosol fractions from THS was significantly lower than that observed for the 3R4F reference cigarette aerosol fractions.

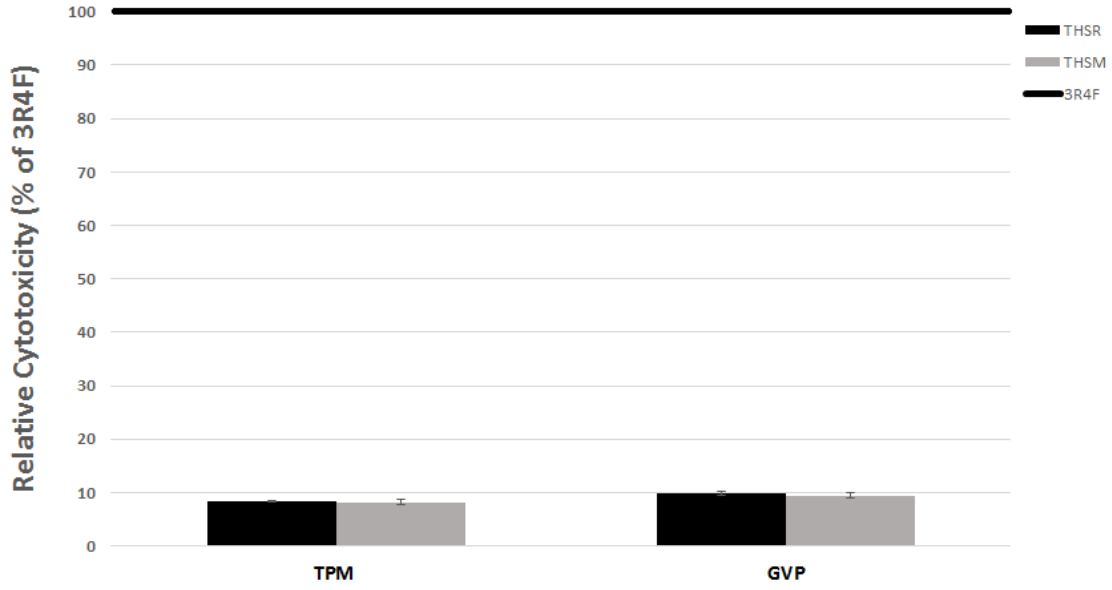


Figure 1 **Cytotoxicity (1/EC50) Relative to 3R4F of THSR and THSM on a per mg Nicotine Basis. Means ± SEM**

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Table 2 1/EC50 of 3R4F of THSR and THSM on a per mg Nicotine Basis. M= mean; SE = standard error.

Cytotoxicity THSM		Stat. Parameter	TPM	GVP	Cytotoxicity 3R4F		Stat. Parameter	TPM	GVP
$1/EC_{50}$ (ml/mg)		M	19.73	26.07	$1/EC_{50}$ (ml/mg)		M	239.51	276.21
		SE	0.87	1.78			SE	6.07	22.51
Cytotoxicity THSR		**Stat. Parameter**	**TPM**	**GVP**	**Cytotoxicity 3R4F**		**Stat. Parameter**	**TPM**	**GVP**
$1/EC_{50}$ (ml/mg)		M	17.34	28.4	$1/EC_{50}$ (ml/mg)		M	208.55	289.06
		SE	0.52	1.2			SE	6.92	22.38

6.1.2.2.2 Bacterial Mutagenicity (AMES) Assay

6.1.2.2.2.1 Introduction

Mutagenicity is mechanistically strongly related to carcinogenicity (Klaunig and Kamendulis, 2008; Preston and Hoffmann, 2008). Mutations to DNA are usually linked to the initiation stage in multi-stage carcinogenesis models. Indeed, though mutations may also occur at later stages in carcinogenesis, for the action of many carcinogens, mutations represent a necessary first step, in particular if occurring in proto-oncogenes and/or tumor suppressor genes. Actually, mutational alteration of proto-oncogenes can lead to overexpression of their growth-stimulating activity, whereas mutations that inactivate tumor suppressor genes can release cells from their growth- or proliferation-inhibiting activity. For these reasons, mutagenicity testing is part of practically all regulatory guidance's on risk assessment.

The Ames Salmonella/microsome mutagenicity assay is a short-term bacterial reverse mutation assay specifically designed to detect a wide range of chemical substances that can produce genetic damage that leads to gene mutations (Maron and Ames, 1983). Different *Salmonella typhimurium* strains have been created that can detect different types of mutations. In particular, these bacteria can be used to easily detect base-pair mutations as well as frame-shift mutations and both types of mutations have shown to occur upon smoke exposure (US Department of Health and Human Services, 2010). Actually, base-pair or point mutations have been identified in oncogenes and mutated tumor suppressor genes, and deletions related to frame-shift mutations have also been identified at various chromosomal sites in lung cancer tissues of smokers (US Department of Health and Human Services). Over the years, modifications to the standard Ames test (the plate incorporation assay version) have been developed in order to enhance the sensitivity of the test and to allow the testing of a wider range of chemicals and complex mixtures. The most commonly used modifications are the pre-incubation and the microsuspension procedures. In the preincubation assay, the tester strains are exposed to the chemical for a short period and in a small volume of either buffer or S9 metabolic activation system (an aroclor-induced liver microsome fraction from rat), prior to plating. It is believed that the pre-incubation assay is more sensitive

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than the plate incorporation assay, because short-lived mutagenic metabolites may have a better chance of reacting with the tester strains in the small volume of preincubation mixture, and the effective concentration of S9 mix in the preincubation volume is higher than on the plate. This test version has been integrated in a dedicated OECD guideline (OECD 1997). One additional modification to the standard method is the microsuspension procedure that was specifically designed to detect mutagenic metabolites in relatively small sample volumes (e.g. urine samples obtained from animals treated with test chemicals) (Kado et al., 1983). In this procedure, the cultures are concentrated to obtain a 10-fold higher than normal cell density and then used in the preincubation assay. The exposure of a higher number of bacteria to the samples is believed to enhance the detection of mutagenic metabolites. However, the microsuspension procedure is not considered as an alternative to the preincubation method in the official OECD guideline 471. Today, the Ames test is used world-wide as an initial screening to determine the mutagenic potential of new chemicals and drugs and it is frequently used, in combination with mammalian cell genotoxicity assays, for submission of data to regulatory agencies. The combination of bacterial and mammalian genotoxicity assays is important because it considerably reduces the likelihood of obtaining false negative or false positive results linked to the intrinsic limitations of single tests such as the nature of the test system.

In the tobacco industry, the Ames assay has long been used for the assessment of mutagenic activity and potency of tobacco smoke. In particular, thanks to its reproducibility (Roemer et al., 2002; Oldham et al., 2012; CORESTA *In vitro* Toxicology Task Force, 2007; Rickert et al., 2007), discriminatory power (Oldham et al., 2012; (CORESTA *In vitro* Toxicology Task Force, 2007) and high predictive value (Tennant et al., 1987; Pfuhler et al., 2010; Kirkland et al., 2011; Benigni, 2012), the Ames assay has been used to differentiate the mutagenic potential of smoke from different tobaccos. Altogether, the Ames assay has demonstrated its efficacy and as such it is deemed suitable to be incorporated into the *in vitro* test battery for the comparative assessment of mutagenic activity of THS aerosol versus that of CC smoke.

6.1.2.2.2.2 Methods

The Ames assay was performed in accordance to the OECD Guideline for the Testing of Chemicals, TG 471 (OECD, 1997). The study quality standards followed the OECD GLP principles. The assay employs several histidine dependent Salmonella strains, each carrying different mutations in various genes in the histidine operon. These mutations act as hot spots for mutagens that cause DNA damage via different mechanisms. When the Salmonella tester strains are grown on a minimal media agar plate depleted of histidine, only those bacteria that revert to histidine independence (his+) are able to form colonies. However, when a mutagen is added to the plate, the number of revertant colonies per plate is increased, usually in a dose-related manner. As these bacteria do not have a metabolic system resembling that from eukaryotic organisms, the S9 metabolizing fraction is incorporated in the test to detect those compounds that require metabolic activation to become mutagenic.

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For the test performed with 3R4F, THSR and THSM, five *Salmonella typhimurium* strains (TA98, TA100, TA102, TA1535, and TA1537) both with and without an S9 metabolizing fraction were used. In each test, concurrent positive, negative and solvent controls with and without S9, were evaluated and used to confirm the test performance. Only the TPM fractions from the aerosols were assayed since they represent the main driver of the mutagenic effects in the Ames test whereas GVP showed only limited responses (Aufderheide et al., 2008; Zenzen et al., 2012; Weber et al., 2010). A preliminary dose range finding assay was performed with the THS TPM aerosol fraction in order to define the maximum doses tolerated by the test system. Each substance concentration (control, reference or test item) was tested in triplicate and on two separate test occasions. The THS-aerosol was regarded as mutagenic (i.e. positive) in the Ames test if a reproducible 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, has occurred (OECD, 1997). Increases of at least 2-fold in the number of revertants above background were considered as biologically relevant for TA98, TA100 and TA102 and 3-fold for TA1535 and TA1537.

6.1.2.2.2.3 Results and Conclusion

In these studies (for THSR: study RLS-ZRH-2015-253 and for THSM: study RLS-ZRH-2015-254), all bacterial strains used showed the expected response with the applied positive control substances (4-Nitro-o-phenylenediamine, Sodium Azide, Cumene Hydroperoxide, and 9-Aminoacridine for the –S9 arm and Benzo(a)pyrene and 2-aminoanthracene for the +S9 arm). Results were reported on a per mg nicotine and per stick basis.

In the bacterial strains TA98, TA100 and TA1537, reproducible mutagenic responses were observed for the TPM from 3R4F in the presence of S9. In contrast, no biologically relevant mutagenicity was found with TA102 or TA1535 in the presence of S9 given that treatment-associated increases in revertant colony numbers were below two times the vehicle controls. No biologically relevant mutagenicity was found in any strain for the TPM from 3R4F in the absence of S9

The TPM from THSR and THSM was tested in TA98, TA100, TA1537, TA102 and TA1535 in the presence or absence of S9 and not found to be mutagenic.

In conclusion, under the assay conditions used, TPM from THSR and THSM did not show any mutagenic activity in the different strains tested irrespective of the presence or absence of S9.

6.1.2.2.3 Mammalian Cell Mutagenicity (Mouse Lymphoma Assay)

Mammalian cell genotoxicity and mutagenicity tests are usually used in a battery to complement the bacterial mutation assay in a first tier of *in vitro* genotoxicity testing for regulatory purposes (Dybing et al., 1997; Cimino, 2006), because no single test is capable of detecting all classes of genotoxic compounds and all types of genotoxic effects. For these reasons, genotoxicity testing is a central part of risk assessment procedures used for industrial chemicals, pharmaceutical drugs, and consumer products. Similarly, several

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authoritative organizations recommend using genotoxicity testing for tobacco samples (Health Canada, 2005; UK Department of Health, 1998; UK Committee on Mutagenicity of Chemicals in Food Consumer Products and the Environment, 2011) or pharmaceuticals (e.g., ICH, 1997). To assess genotoxicity and/or mutagenicity, different endpoints must be taken into considerations: beside point mutations induction, a compound can induce changes in chromosomal number (polyploidy or aneuploidy) or in chromosome structure (breaks, deletions, rearrangements). For that reason, a range of relevant internationally agreed testing methods is available including the Mouse Lymphoma Assay (MLA), *In Vitro* Micronucleus Assay (MNvit), Comet assay, Chromosomal Aberrations Assay, etc. Although all these methods have been explored by tobacco companies to assess the genotoxic potential of tobacco products historically, the MLA has been the method of choice to assess mutagenicity in mammalian cells (Combes et al., 2012; Schramke et al., 2006; Werley et al., 2008). This is due to its ability to differentiate between samples of tobacco products, such as smoke of different cigarette types, e.g., for the assessment of manufacturing processes, tobacco varieties, and novel tobacco products. The assay has been technically qualified in order to evaluate the intra- and inter-study reproducibility and the discriminatory power (Combes et al., 2012; Guo et al., 2011; Schramke et al., 2006; Oldham et al., 2012). In addition, over the past years, an increasing number of academic, contract, and governmental researchers have also recommended or used the MLA to assess the *in vitro* genotoxicity of tobacco smoke (e.g., Albert, 1983; Clive et al., 1979; Guo et al., 2011; Mitchell et al., 1981).

The Mouse Lymphoma Assay (MLA) has the potential to detect mutagenic and clastogenic events at the thymidine kinase (tk) locus of L5178Y mouse lymphoma tk +/− cells by measuring resistance to the lethal nucleoside analogue triflurothymidine. When the cells are added to selective medium containing triflurothymidine, wild-type tk +/− cells die, but triflurothymidine cannot be incorporated into the DNA of mutant tk −/− cells, which survive to form colonies that may be large (indicative of gene mutation) or small (indicative of gross chromosomal damage) in nature. In the standard protocol, L5178Y mouse lymphoma tk +/− cells are exposed to the test substances with and without metabolic activation for a defined period of time, usually 4 h and 24 h. Continuous treatment without metabolic activation for 24 h is needed especially when negative results are obtained for the short treatment without metabolic activation. This is based upon the enhanced detection of a number of nucleoside analogues and base analogues test substances when a 24 h treatment is included. Similarly, the detection of aneuploidy inducers was stated to be enhanced when using 24 h treatments, though the experimental evidence for the detection of aneuploidy was noted to be limited (Clements et al., 2000).

Many authoritative organizations advise the use of the MLA because the assay has demonstrated its ability to detect most of the mutational events known to be associated with the etiology of cancer and other human diseases, including point mutations and a number of different types of chromosomal damage, as well as recombination and deletion (Zeiger, 2010; Applegate et al., 1990; Chen et al., 2002; Clive et al., 1995; Honma et al., 2001; Liechty et al., 1998, Wang et al., 2009). Altogether, the MLA is widely accepted in

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many areas of risk assessment because of its accuracy, reproducibility, discriminatory power and biological relevance for certain smoking-related diseases. For these reasons, the MLA is considered a fundamental part of a battery of *in vitro* assays for the assessment of the genotoxic potential of the THS aerosol in a weight-of-evidence evaluation of potential for risk reduction.

6.1.2.2.3.1 Methods

The MLA was performed in accordance with the OECD Guideline for the Testing of Chemicals Protocol TG 490 (OECD, 2016). The study quality standards followed the OECD GLP principles. For the completion of this study, two independent tests were performed for each of the three treatment conditions, 4 h ±S9 and 24 h -S9.

Briefly, L5178Y mouse lymphoma cells were exposed in separate experiments to TPM and GVP derived from the aerosol of the test items, THSR and THSM, and from the smoke of 3R4F reference item. Cytotoxicity data, i.e. relative total growth (RTG), and mutagenicity data, i.e. mutation frequency (MF), were generated and used to determine the *in vitro* mutagenicity of test item-derived aerosol fractions under 4 h ±S9 and 24 h -S9 treatment conditions in two independent tests and to compare their lowest observable genotoxic effect levels (LOGELs) (Guo et al, 2016) with those of counterpart fractions derived from the reference item. TPM- and GVP-treated cells which underwent excessive cytotoxicity, i.e. Relative Total Growth (RTG) <10%, were discarded at their respective stage in the assay procedure as mutagenicity data derived from these cells are difficult to interpret because of their questionable biological relevance. Indeed, excessive cytotoxicity could actually lead to false positive results (OECD, 2016).

6.1.2.2.3.2 Results and Conclusion

Both TPM and GVP aerosol fractions from THSR, THSM and 3R4F (for THSR: study RLS-ZRH-2015-252 and for THSM: study RLS-ZRH-2015-251) induced a concentration-dependent increase in cytotoxicity and mutagenicity. Indeed, although the responses observed were variable between assay replicates (Figure 2 and Figure 3), all aerosol fractions induced a biologically-relevant mutagenic response in at least one of the three treatment conditions (4 h ±S9 and 24 h -S9) as classified by the global evaluation factor (GEF) approach (Moore et al., 2006; Moore et al., 2007). However, the aerosol concentrations at which positive mutagenic responses were induced differed between THS and 3R4F aerosol fractions, indicating a difference in mutagenic potencies *in vitro*. Only at concentration significantly higher did THS 2.2 trigger mutagenic responses.

In particular, on a per mg TPM basis, LOGELs were found to be different between THSR-derived TPM (between 19.4 and 29.6-fold higher) and GVP (between 8.3 and 15-fold higher) compared with LOGELs from counterpart 3R4F aerosol fractions. Similar results were obtained considering the results on a per mg nicotine basis. Indeed, LOGEL values for THSR-derived TPM were between 14 and 21.9-fold higher compared with LOGELs values for 3R4F-derived TPM (Table 3).

Similarly, on a mg TPM basis, LOGELs were found to be different between THSM-derived TPM (between 15.4 and 22.9-fold higher) and GVP (between 14.4 and 23.6 fold higher) compared with LOGELs from counterpart 3R4F aerosol fractions. Comparable

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results were obtained considering the results on a per mg nicotine basis. Indeed, LOGEL values for THSM-derived TPM were between 9.1 and 13.1-fold higher compared with LOGELs values for 3R4F-derived TPM (Table 4).

In conclusion, although the TPM and GVP aerosol fractions derived from THSR and THSM displayed a mutagenic response in the MLA, their mutagenic potency *in vitro* was lower compared with the corresponding fractions derived from 3R4F.

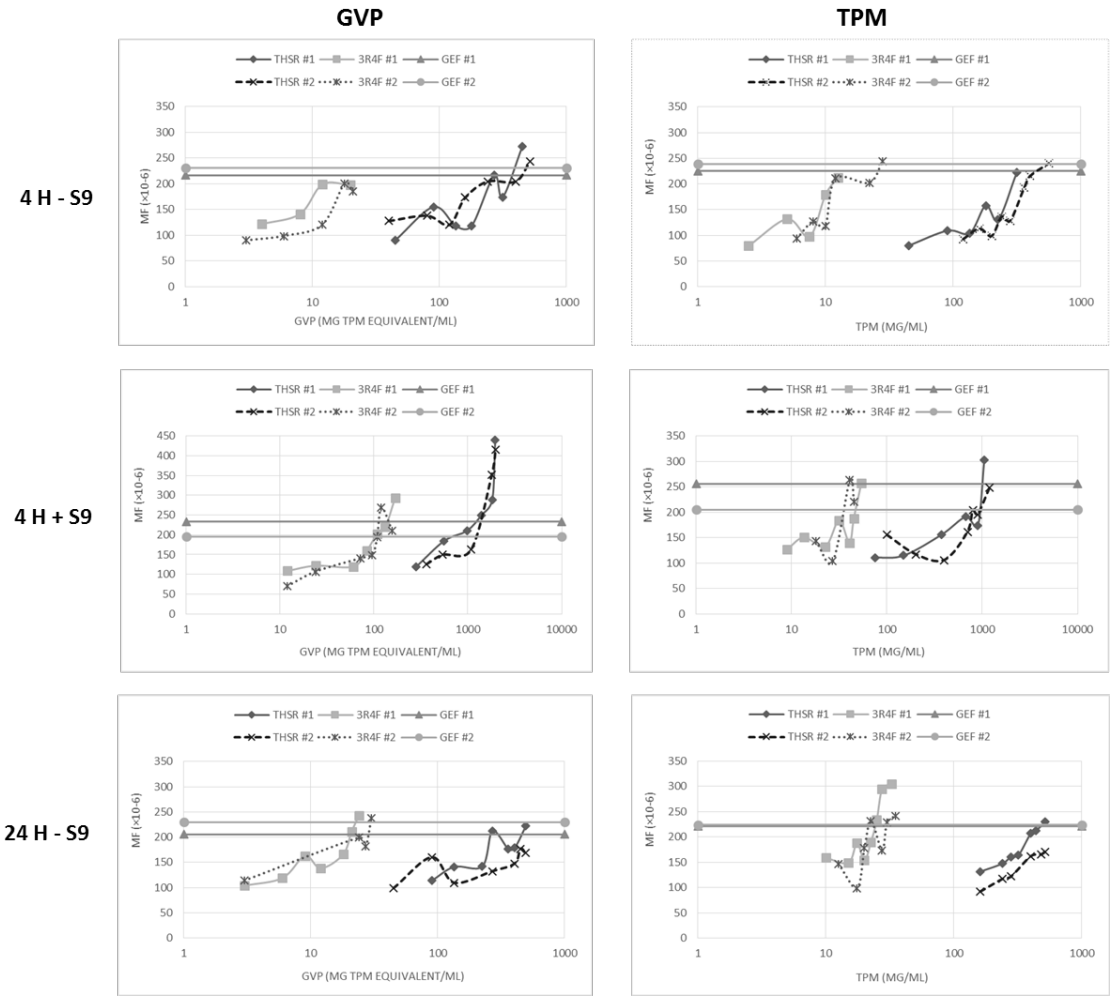


Figure 2 **Mutagenicity in MLA of TPM and GVP of THSR compared with 3R4F Smoked under HCI, TPM equivalent, with and without S9 Metabolic Activation. #1= replicate1; #2 replicate2**

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Table 3 LOGEL values and ratios on a per µg TPM and µg Nicotine basis for THSR and 3R4F. N/A = Not Applicable

			4 h -S9 TPM-induced LOGELs			
Test Number	**Fraction**	**Item**	**LOGEL (µg/ml)**	**LOGEL Ratio**	**Nicotine at LOGEL (µg/ml)**	**Nicotine at LOGEL Ratio**
1	TPM	THSR	N/A	N/A	N/A	N/A
		3R4F	N/A	N/A	N/A	N/A
2	TPM	THSR	560	20	18.66	14.3
		3R4F	28	1	1.308	1
			4 h +S9 TPM-induced LOGELs			
1	TPM	THSR	1050	19.4	34.483	14
		3R4F	54	1	2.467	1
2	TPM	THSR	1200	29.6	41.804	21.9
		3R4F	40.5	1	1.905	1
			24 h -S9 TPM-induced LOGELs.			
1	TPM	THSR	520	20.8	17.31	15.1
		3R4F	25	1	1.143	1
2	TPM	THSR	N/A	N/A	N/A	N/A
		3R4F	22.5	1	1.051	1

			4 h -S9 GVP-induced LOGELs	
Test Number	**Fraction**	**Item**	**LOGEL (µg TPM equivalent/ml)**	**LOGEL Ratio**
1	GVP	THSR	450	N/A
		3R4F	N/A	N/A
2	GVP	THSR	520	N/A
		3R4F	N/A	N/A
			4 h +S9 GVP-induced LOGELs	
1	GVP	THSR	1400	8.3
		3R4F	168	1
2	GVP	THSR	1800	15
		3R4F	120	1
			24 h -S9 GVP-induced LOGELs	
1	GVP	THSR	270	12.9
		3R4F	21	1
2	GVP	THSR	N/A	N/A
		3R4F	30	1

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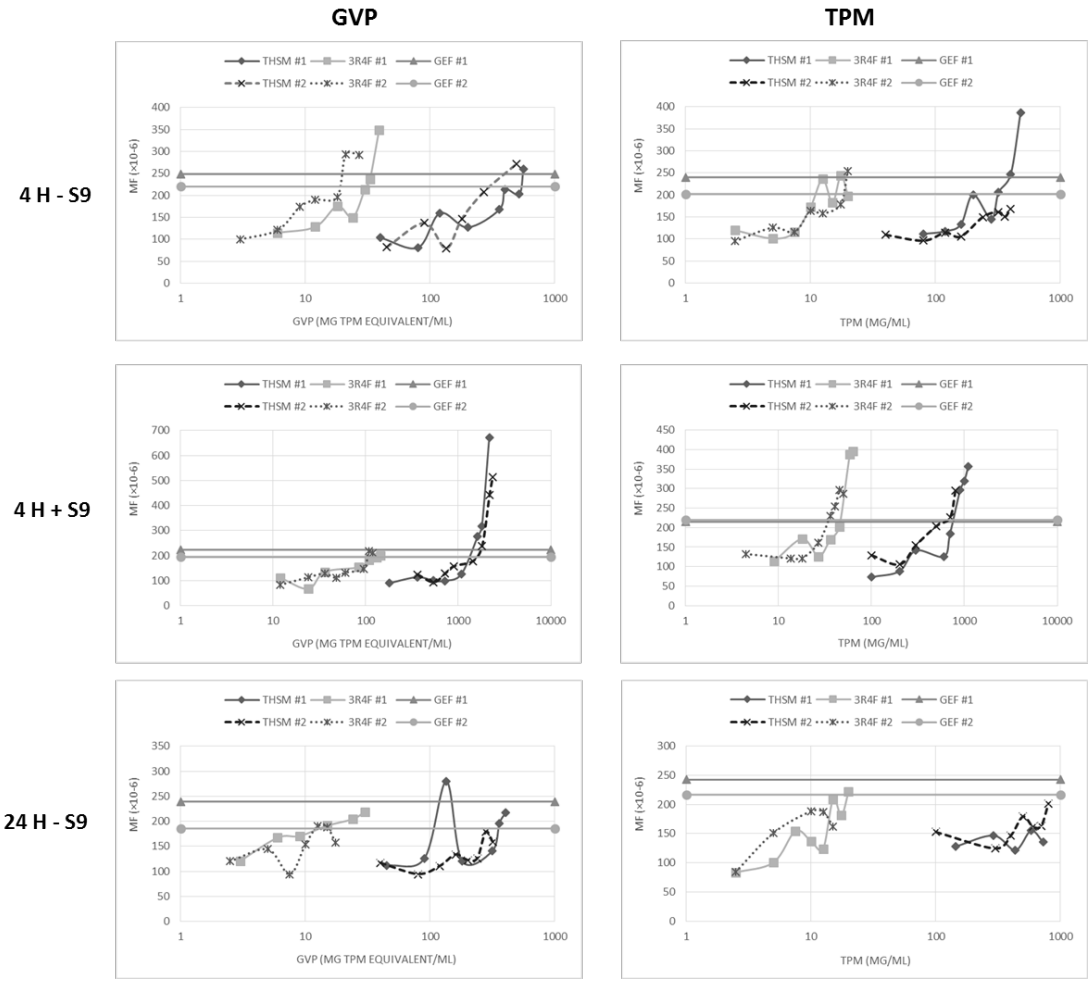


Figure 3 **Mutagenicity in MLA of TPM and GVP of THSM compared with 3R4F Smoked under HCI, TPM equivalent, with and without S9 Metabolic Activation. #1= replicate1; #2 replicate2**

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Table 4 LOGEL values and ratios on a per µg TPM and µg Nicotine basis for THSM and 3R4F. N/A = Not Applicable

			4 h -S9 TPM-induced LOGELs			
Test Number	**Fraction**	**Item**	**LOGEL (µg/ml)**	**LOGEL Ratio**	**Nicotine at LOGEL (µg/ml)**	**Nicotine at LOGEL Ratio**
1	TPM	THSM	400	22.9	12.546	13.1
		3R4F	17.5	1	0.955	1
2	TPM	THSM	N/A	N/A	N/A	N/A
		3R4F	20	1	1.106	1
			4 h +S9 TPM-induced LOGELs.			
1	TPM	THSM	900	15.4	27.817	9.1
		3R4F	58.5	1	3.051	1
2	TPM	THSM	700	19.4	21.706	10.9
		3R4F	36	1	1.99	1
			24 h -S9 TPM-induced LOGELs.			
1	TPM	THSM	N/A	N/A	N/A	N/A
		3R4F	N/A	N/A	N/A	N/A
2	TPM	THSM	N/A	N/A	N/A	N/A
		3R4F	N/A	N/A	N/A	N/A

			4 h -S9 GVP-induced LOGELs	
Test Number	**Fraction**	**Item**	**LOGEL (µg TPM equivalent/ml)**	**LOGEL Ratio**
1	GVP	THSM	560	14.4
		3R4F	39	1
2	GVP	THSM	495	23.6
		3R4F	21	1
			4 h +S9 GVP-induced LOGELs.	
1	GVP	THSM	1620	N/A
		3R4F	N/A	N/A
2	GVP	THSR	1800	16.7
		THSM	108	1
			24 h -S9 GVP-induced LOGELs.	
1	GVP	THSM	N/A	N/A
		3R4F	N/A	N/A
2	GVP	THSM	N/A	N/A
		3R4F	12.5	1

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6.1.2.3 *In vivo* studies

For the *in vivo* pre-clinical toxicity assessment, two 90-day inhalation studies were performed on Sprague Dawley rats, and one 18-month chronic toxicity and carcinogenicity study was performed on A/J mice. Table 5 provides an overview of the studies.

Table 5 Pre-clinical *in vivo* safety toxicology studies

Study type	Protocol	Test System	Test item	Study number
90-day inhalation study	OECD 413	Rat, Sprague Dawley	THS Regular: (b) (4)	15006
90-day inhalation study	OECD 413	Rat, Sprague Dawley	THS Menthol: (b) (4)	15025
Chronic toxicity and cancer inhalation study	OECD 453	A/J mouse	THS Regular: (b) (4) / DORADO II / (b) (4) (b) (4)	15020

6.1.2.3.1 90-day inhalation studies

6.1.2.3.1.1 Introduction

Animal studies are an essential part of risk assessment approaches in toxicology. The FDA Guidance (U.S. FDA CTP, 2012) recommends to include *in vivo* studies in the evidence package for the evaluation of tobacco products with potential for risk reduction. The aim of pre-clinical *in vivo* studies is to ensure that the THS is not more toxic than 3R4F reference cigarette, does produce a meaningful reduction in toxicity compared with 3R4F and, does not produce new potential risks. These data are required before clinical studies using THS can be conducted. In our preclinical assessment strategy, we have included the 90-day rat inhalation study as part of the test battery to substantiate the potential for risk reduction associated with the usage of THS. The inhalation route was selected because it is "the route representative of human exposure" (U.S. FDA CTP, 2012) for THS. The 90-day study duration has been selected because it provides a pragmatic and effective approach between the very short duration studies, which are not compatible with accurate modelling of the long-term processes that are thought to occur in smoke-induced diseases and, life-time duration studies that routinely do not show responses that are different from those seen after a few months of exposure (Coggins et

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al., 1982) and that are in any case becoming increasingly questionable (Gori, 2013; Jacobson-Kram, 2010; Doktorova et al., 2012). The selection of aerosol concentrations to be tested and the daily dose is a critical aspect of inhalation studies with cigarette smoke and THS aerosol is no exception. With smoke from conventional cigarettes there is a built-in maximum for a daily exposure, resulting from the presence of carbon monoxide in the smoke. The carbon monoxide reduces the oxygen-carrying capacity of blood, through the formation of carboxyhemoglobin. Therefore, the highest target nicotine concentrations for reference cigarettes was chosen to avoid daily doses that approach the blood carboxyhemoglobin concentration associated with rat mortality: around 60% (Ayres et al., 1989). THS generates low quantities of carbon monoxide; therefore, the nicotine toxicity itself and not exposure to high levels of carbon monoxide represent the dose-limiting factor for THS dose selection.

6.1.2.3.1.2 Methods

Two independent *in vivo* subchronic 90-day inhalation studies, one using THSR (study 15006) and one with THSM (study 15025), were performed in accordance with OECD guideline 413 (OECD, 2009a).

Sprague-Dawley rats were nose-only exposed to three target concentrations of either 3R4F reference cigarette smoke or THS aerosol for six hours/day, five days/week for thirteen weeks. A control (sham) group exposed to conditioned air under the same experimental conditions was included in both studies. Furthermore, in the study conducted with THSM, two mentholated CCs were included as additional references. As THSM was designed to yield 1.2 mg/stick menthol in smoke (MIS) when smoked according to ISO standard 3308 (ISO, 2000), the two mentholated reference CCs were produced by PMI to the 3R4F specifications and designed to also yield 1.2 mg/stick (DDA3 1XMIS) and 2.4 mg/stick MIS (DDA3 2XMIS). These menthol levels were chosen to be in the range of those in the aerosol from THSM.

The 3R4F cigarette smoke and THS aerosol were generated according to the Health Canada Intensive Smoking Protocol (Health Canada, 1999) which is based on the ISO standard 3308 (ISO, 2000) with the exceptions of the puff volume (55ml), puff duration (2 seconds), puff frequency (one puff every 30 seconds) and closing off ventilation holes where applicable. Prior to the main study, a dose range finding study was performed to provide a rationale for the selection of concentrations for the main study.

In both main studies, the target concentrations of 3R4F smoke and THS aerosol were adjusted to deliver test atmosphere nicotine concentrations of 8, 15, 23 µg/l and 15, 23 and 50 µg/l respectively. This ensures that the highest nicotine concentration of the THS aerosols was twice as high as the highest smoke nicotine concentrations from the reference cigarettes. Satellite study groups were exposed to the aerosol from THS at the highest concentration level for 13 weeks followed by 42 days recovery period in order to investigate the reversibility or persistence of test substance related effects. Based on OECD Testing Guideline 413 and previous experiments on the same endpoints (Terpstra et al., 2003; Vanscheeuwijck, 2000; Vanscheeuwijck et al., 2002), the sample size was fixed to 10 male and 10 female rats per group for all OECD groups with the

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exception of the Control group (sham) and 3R4F high (23 µg/l nicotine) recovery groups where only 6 male and 6 female rats per group were allocated. In addition, a subset of animals (PLUS groups) in each exposure concentration was dedicated to provide biological samples for further analysis using a systems toxicology-based approach (section 7.5.2.5 of 7.5 Summary).

To confirm the appropriate exposure of the animals, the smoke and aerosol test atmospheres were monitored by on-line carbon monoxide measurements and daily determinations of both nicotine and total particulate matter (TPM) concentrations in samples taken from the breathing zone of the exposure chambers. Biomonitoring was performed through both blood carboxyhemoglobin measurements and the quantification of the main nicotine metabolites in urine and/or in blood samples (section 6.1.3.2.4 and Appendix A6.1.3.26).

The endpoints for analysis, as outlined in the OECD Testing Guideline 413 for sub-chronic inhalation studies, included clinical examination during and following exposure. Parameters investigated were health status, body weight, ophthalmoscopy and food consumption, pathology (post-mortem gross necropsy and histopathological assessment of both respiratory and non-respiratory organs and tissues), urinalysis, blood analysis (clinical chemistry, complete blood counts), and in addition, bronchoalveolar lavage fluid (BALF) analysis. The respiration of the rats was monitored using indirect plethysmography.

6.1.2.3.1.3 Results and Conclusion

Aerosol generation was reproducible throughout the exposure period and the THSR and THSM aerosols were demonstrated to be efficiently taken up by the animals as indicated by the biomonitoring parameters. Results of carboxyhemoglobin, nicotine and other urinary aerosol constituent metabolites correlated well with the constituent concentrations in the test atmospheres (Appendix A6.1.3.26).

In the first 90-day study (study 15006, THSR) For equal test atmosphere nicotine concentrations, nicotine uptake of THSR was higher than that of 3R4F because of the lowered respiratory frequency and consequently respiratory minute volume in 3R4F-exposed rats. This lower respiratory frequency is due to irritation of the upper respiratory tract and it is mediated by the trigeminal nerve. In the THSR-exposed rats, the clinical (in-life) observations were similar than those observed in 3R4F-exposed rats, but in general, their severity was lower. The biological effects from the test and reference items were limited mainly to hematological changes (increased neutrophil counts), changes in blood chemistry (rise in liver enzyme activities, reduced total protein, albumin and triglycerides in females, reduced cholesterol), and in organ weights (notably in lungs, liver, adrenal glands, thymus, uterus) (Table 6). Histopathological changes including inflammation seen in the respiratory tract organs of 3R4F-exposed animals were adaptive and degenerative changes that were within historical ranges upon exposure to conventional cigarette smoke. Histopathological changes observed in the 3R4F-exposed animals were either not observed or their severity was significantly lower in the THSR-exposed rats (Table 6). Most changes induced by aerosol exposure in the respiratory tract

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organs showed reversal of effects at the end of the post-inhalation (recovery) period. Interestingly, the findings observed in THSR-exposed rats were very similar to those observed in other *in vivo* inhalation studies using nicotine-exposed rats suggesting that most effects were associated with nicotine exposure itself (Phillips et al., 2015). Most findings in the non-respiratory tract organs were considered normal background changes in Sprague Dawley rats. Several minimal exposure-related effects were found, including more mast cells and yellow pigmentation in mesenteric lymph nodes of 3R4F and THSR exposed animals, lower mononuclear cell infiltration in livers of female 3R4F and THSR exposed animals, thymus atrophy in 3R4F and THSR exposure groups.

In a second study (study 15025, THSM), the biological effects of inhaled aerosol from THSM in comparison with mainstream smoke from 3R4F and two mentholated reference cigarettes DDA3 1XMIS (1.2 mg menthol in smoke) and DDA3 2XMIS (2.4 mg menthol in smoke) was evaluated. The respiratory frequency across THSM groups was similar to that of Sham-exposed animals and at equal test atmosphere concentrations of nicotine, THSM groups had higher nicotine and nicotine metabolites recovered in blood and urine reflecting the lower irritant capacity of the test aerosols compared with 3R4F. Clinical (in-life) observations made in THSM-exposed rats were comparable with those in rats exposed to smoke from 3R4F/ DDA3 1XMIS / DDA3 2XMIS, but in general their severity was lower. The biological effects from the test and reference items were mostly related to hematological changes (increased neutrophil counts), changes in blood chemistry (rise in liver enzyme activities, reduced total protein, albumin and cholesterol), and in organ weights (notably in lungs, livers, adrenal glands, thymus, uterus) (Table 6). The low free lung cell counts and BALF inflammatory analytes in rats exposed to THSM aerosol point towards a lower degree of respiratory tract irritation and inflammation. The reduced toxicity of THSM aerosols on the respiratory tract organs was further confirmed by histopathological analysis of the respiratory tract organs. Histopathological changes, including inflammation seen in the respiratory tract organs of 3R4F/ DDA3 1XMIS / DDA3 2XMIS-exposed animals, were adaptive and degenerative in nature and within historical ranges based on results from previously conducted nose only CC smoke inhalation studies on rats. Those changes were either not observed or significantly lower in severity in the THSM-exposed rats. Most changes induced by aerosol exposure in the respiratory tract organs showed reversal of effects at the end of the post-inhalation (recovery) period. Most findings in the non-respiratory tract organs were considered normal background changes in Sprague Dawley rats. Overall, the data suggest that exposure to THSM aerosol produces less inflammation and degenerative changes to the respiratory tract organs and does not result in additional hazards to those presented by smoking conventional cigarettes. In addition, no remarkable effects were noted due to the added levels of menthol and similar results were observed in the 3R4F/ DDA3 1XMIS / DDA3 2XMIS-exposed rats.

Overall, the data from the 90-day inhalation studies shows that exposure to THS aerosols, irrespective of the addition of menthol, produces less inflammation and degenerative changes compared with reference cigarette, even at much higher test atmosphere nicotine concentrations. Moreover, most findings in both respiratory and non-respiratory tract organs were very similar to those observed in previous published studies where animals

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were exposed to nicotine and nicotine salt solutions at equivalent nicotine test atmosphere concentration (Phillips et al., 2015) suggesting that most of effects observed are most likely due to the nicotine.

Table 6 Summary of systemic toxicity and histopathology of male and female rats exposed to smoke from 3R4F and aerosol from THSR and THSM in the 90-day inhalation studies

Biological Endpoints	3R4F	THSR (15006)	THSM (15025)
MORTALITY	No death related to exposure up to 23 µg/L nicotine in test atmosphere.	No death related to exposure up to 50 µg/L nicotine in test atmosphere.	No death related to exposure up to 50 µg/L nicotine in test atmosphere.
BODY WEIGHT	Body weight dropped slightly during the first week of dose adaptation. At the end of exposure, ~ 10 % lower compared with sham.	Body weight dropped slightly during the first week of dose adaptation. A significant reduction in body weight was observed only in male THSR-exposed animals when compared with sham. At equivalent nicotine concentration, mean body weight of 3R4F High was lower than THSR Medium. At the end of exposure, body weights of male rats was ~ 10 % lower than sham. Female rats showed a higher body weights when compared with sham.	Reduced body weight gain observed only in male animals but less pronounced when compared with 3R4F. Female groups showed a higher body weight when compared with sham.
RELATIVE/ABSOLUTE ORGAN WEIGHTS	Higher lung, larynx and trachea weight compared with sham.	Higher lung, larynx and trachea weight compared with sham but less pronounced than 3R4F exposed group.	Higher lung, larynx and trachea weight compared with sham but less pronounced than 3R4F exposed group.
	Higher liver weights compared with sham.	Higher liver weights compared with sham.	Higher liver weights compared with sham.
	Higher adrenal gland weight compared with sham.	Higher adrenal gland weight compared with sham.	Higher adrenal gland weight compared with sham.

(table continues)

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Biological Endpoints	3R4F	THSR (15006)	THSM (15025)
	Lower absolute and relative thymus weights compared with sham.	Male animals: Lower absolute and relative thymus weights compared with sham but less pronounced or comparable to 3R4F.	Lower absolute and relative thymus weights compared with sham but less pronounced or comparable to 3R4F.
	Lower weight of spleen compared with sham.	THSR aerosol induced reduction in relative spleen weights in females compared with sham.	THSM aerosol induced reduction in spleen weights compared with sham.
	Lower uterus weight compared with sham.	Lower uterus weight compared with sham.	Lower uterus weight compared with sham.
RESPIRATORY PHYSIOLOGY	Lower respiratory minute volume compared with sham.	No change in respiratory minute volume compared with sham.	Respiratory frequency similar to that of sham.
LUNG INFLAMMATION	Concentration-dependent increased in total immune cell count compared with sham.	Marginal increase in immune cell count present in bronchoalveolar lavage fluid compared with sham.	No significant changes in immune cell counts present in bronchoalveolar lavage fluid compared with sham.
CLINICAL CHEMISTRY	Higher liver enzyme activity compared with sham.	Higher liver enzyme activity compared with sham. Effects similar to 3R4F.	Higher liver enzyme activity compared with sham. Effects similar to 3R4F.
	Lower level of triglycerides compared with sham.	Lower level of triglycerides compared with sham.	Lower level of triglycerides compared with sham.
HISTOPATHOLOGY OF THE RESPIRATORY TRACT ORGANS	Nose	Nose	Nose
	Hyperplasia and squamous epithelial metaplasia of the respiratory epithelium at nose level 1 and 2 (moderate to marked/massive severity), atrophy of olfactory epithelium at nose level 2 (mild to moderate severity), loss	Changes in THSR-exposed animals were observed mostly in nose level 1 and absent in nose levels 3 to 4. At nose level 1, reseve cell hyperplasia (minimal to moderate severity) and squamous epithelial metaplasia (minimal to moderate severity)	Changes in THSM-exposed animals were observed mostly in nose level and absent in nose levels 3 to 4. Hyperplasia of the respiratory epithelium at nose level 1 (minimal to mild severity), at highest exposure

(table continues)

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Biological Endpoints	3R4F	THSR (15006)	THSM (15025)
	of nerve bundles at level 2, 3 and 4 (minimal to moderate severity), presence of amorphous eosinophilic material in the respiratory region at nose levels 1, 2 and olfactory region nose level 4 (minimal to moderate).	were noted. The extend of corninication was minimal or absent compared with 3R4F.	concentration.
	Larynx	Larynx	Larynx
	Squamous cell metaplasia at the base of epiglottis (mild to marked/severe severity) together with keratosis (minimal to mild severity) were noted . At the arytenoids, squamous cell metaplasia (mild to massive/severe severity) often associated with keratosis was noted. In the ventral pouch, basal cell hyperplasia (moderate severity) together with squamous cell metaplasia (minimal to moderate severity) and keratosis (minimal to mild severity) was observed. At the mid lateral vocal folds, basal cell hyperplasia and squamous cell metaplasia (minimal to moderate severity) and keratosis were noted. At the dorsal vocal fold, basal cell	In THSR-exposed animals, reserve cell hyperplasia at mid and distal base of epiglottis was observed (moderate to severe) and at pseudostratified epithelium of vocal folds was observed instead of squamous epithelial metaplasia that was seen in 3R4F-exposed animals. Similarly, hyperplasia at floor of arytenoid projections was observed in THSR-exposed animals (minimal severity) instead of squamous epithelial metaplasia that was seen in 3R4F-exposed animals.	Basal cell hyperplasia (mild severity) and squamous cell metaplasia (minimal to moderate severity) were observed in association with keratosis (minimal to mild severity). At the arytenoids basal cell hyperplasia (minimal severity) and squamous cell metaplasia (minimal to mild severity) without evident signs of keratosis. In the ventral pouch, basal cell hyperplasia (minimal to mild severity) and squamous cell metaplasia (mild to moderate severity) were noted and minimal keratosis. At the mid lateral vocal folds, basal cell hyperplasia (minimal to mild severity) and squamous cell metaplasia (mild to moderate severity) and keratosis were noted. At the dorsal vocal fold, no relevant or

(table continues)

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Biological Endpoints	3R4F	THSR (15006)	THSM (15025)
	hyperplasia and squamous cell metaplasia were noted (minimal to mild severity). At the lateral floor, basal cell hyperplasia (mild severity) and squamous cell metaplasia were observed (mild to marked severity), keratosis (minimal to moderate severity)		incidental findings were recorded for THSM. At the lateral floor, basal cell hyperplasia (minimal to moderate severity) and squamous cell metaplasia (minimal to mild severity) were observed with minimal keratosis.
	Tracheal ring and bifurcation	Tracheal ring and bifurcation	Tracheal ring and bifurcation
	At the tracheal ring, basal cell hyperplasia (minimal to mild severity) and goblet cell hyperplasia (minimal to moderate severity) were observed. At the tracheal bifurcation basal cell hyperplasia and goblet cell hyperplasia (minimal to moderate) were observed.	At the tracheal ring and bifurcation, no relevant or incidental findings were recorded for THSR.	At the tracheal ring and bifurcation, no relevant or incidental findings were recorded for THSM.
	Left lung	Left lung	Left lung
	Presence of unpigmented and pigmented macrophages and pigmented macrophage nests (minimal to moderate severity). Presence of neutrophils (minimal severity). Goblet cell metaplasia and hyperplasia typeII (minimal to mild severity) in the bronchus.	In the THSR-exposed animals, macrophages with and without yellow pigmentation in the alveolar lumen were statistically lower (minimal severity) compared with 3R4F as well as the presence of neutrophilic granulocytes. Similarly, goblet cell hyperplasia at the main bronchus was lower in THSR-exposed animals compared with 3R4F.	In the THSM-exposed groups, all observed findings were of similar incidence and/or severity to the sham group, and were considered incidental.

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6.1.2.3.2 Chronic Toxicicity/Carcinogenicity study

6.1.2.3.2.1 Introduction

An 18-month *in vivo* inhalation study has been performed on A/J mice to assess the chronic toxicity of exposure to THS aerosol, and to compare it with the toxicity of smoke from reference cigarette 3R4F. This mouse model has previously been shown to be susceptible to develop lung tumors and has been used in many carcinogenicity studies. In the past, a number of A/J chronic mouse inhalation studies showed increased lung tumor incidence and multiplicity upon exposure to smoke (Stinn et al., 2005; Stinn et al., 2010). Although this mouse strain is developing lung tumors spontaneously, an 18-month inhalation period has been found to be suitable for the development of malignant lesions upon exposure to 3R4F smoke (Stinn et al., 2013a). Smoke-induced tumors can be separated from spontaneously developing tumors using mRNA profiling, based on a 50-gene signature (Luettich et al., 2014). In addition, the A/J mouse strain has been demonstrated to be suitable for assessing effects of chronic inflammation and associated lung emphysema (Stinn et al., 2013b).

The study was designed based on the OECD testing guideline (TG) 453 (OECD, 2009b) for the combined assessment of chronic toxicity and carcinogenicity of chemicals and adapted for exposure of substances via the inhalation route. The duration of the exposure has been targeted to be 18 months because this time period was shown to be feasible for the A/J strain, would allow for distinction between tumor incidences and types in different groups. It should be noted though that spontaneous mortality started to increase after 12 months of exposure or age (Stinn et al., 2013a).

The 18-month inhalation period was completed in June 2016, therefore data analysis is still ongoing. Here we report on the systemic toxicity endpoints, as well as lung inflammation, lung function, emphysema, and histopathology of respiratory tract organs obtained from mice necropsied at interim dissection time points after 1, 5, and 10 months of inhalation exposure to aerosol from THS or smoke from the reference cigarette 3R4F. The dataset is thus not complete, however the data available in this application is prepared and compiled in a draft QA-checked report (see section 7.2 study 15020). This dataset is sufficient to evaluate long term toxicity but not life time toxicity, however it is insufficient to assess the lung tumor incidence and multiplicity.

6.1.2.3.2.2 Methods

The study was carried out in conformity with the OECD TG 453 (OECD, 2009b).

In an 18-months inhalation study (study 15020), A/J mice were exposed to aerosol from THSR at three test atmosphere concentrations of nicotine (6.7 (LOW), 13.4 (MEDIUM) and 26.8 (HIGH) µg nicotine/l test atmosphere) or to one concentration of smoke from 3R4F (13.4 µg nicotine/l test atmosphere) in whole body inhalation chambers (described in Phillips et al., 2016). The 3R4F cigarette smoke and THS aerosol were generated according to the Health Canada Intensive Smoking Protocol (Health Canada, 1999) which is based on the ISO standard 3308 (ISO, 2000) with the exceptions of the puff volume (55ml), puff duration (2 seconds), puff frequency (one puff every 30 seconds) and closing off ventilation holes where applicable. The exposure concentration selection

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was based on previously conducted studies with smoke from 3R4F (Stinn et al., 2013a; Stinn et al., 2013b) that demonstrated the increase in smoke exposure-related lung tumor incidence and multiplicity at a concentration of 300 µg TPM/l test atmosphere, corresponding to 13.4 µg nicotine/l, after 18-months of exposure. In order to assess lung tumor incidence and the chronic toxicity of THS aerosol in comparison with 3R4F smoke, the same exposure concentration was chosen. Furthermore, two groups of mice were exposed to respectively half and twice this concentration to assess the effect of THS aerosol dose. Exposures were carried out for 6 hours per day, 5 days per week. Female mice were exposed to fresh air (Sham), to three concentrations of THS aerosols and one of 3R4F smoke, and necropsies carried out after 1, 5, 10 and 18 months of inhalation exposure. Male mice were exposed to either fresh air (Sham) or to the high test atmosphere concentration of THS aerosol for 18 months. This choice was made to apply the principle of the 3Rs in animal research and based upon previous studies that have shown no difference in incidence and tumor multiplicity between the two genders of A/J mice following smoke inhalation (Stinn et al., 2013a).

The study was powered based on previous experience for the different endpoints investigated, and the requirement from the OECD TG 453 to conclude the study at the 18-month time point of the carcinogenicity phase of the study with at least 50 animals of each group. At the start of the study, 1105 female and 341 male mice were allocated to the different treatment groups and endpoints (including reserve and health check animals).

Test atmospheres were characterized by quantification of a selected number of aerosol constituents during exposure and inside the exposure chambers. Aerosol uptake/exposure was monitored by regularly determining metabolites of aerosol constituents in blood and/or urine.

Chronic toxicity and carcinogenicity endpoints were assessed. The general health condition of the mice was assessed using parameters such as clinical (in-life) observations, body weight, and water and food consumption. During the exposure phase, urinalysis and urine clinical chemistry analysis were performed. At dissection, pathology (post-mortem gross necropsy and histopathological assessment of both respiratory and non-respiratory organs and tissues) and blood analysis (complete blood counts and clinical chemistry) were evaluated. In addition, to gain insight into inflammation and emphysema development in the lung, broncho-alveolar lavage fluid (BALF) analysis, lung function tests and lung morphometric analyses were performed in selected exposure sub-groups. Table 7 shows an overview of the (main) different endpoints investigated after 1, 5 and 10 months of exposure. Additionally, organs and blood were collected for non-OECD Omics endpoints (proteomics, transcriptomics, genomics, and lipidomics) for further analysis using systems toxicology-based approaches to determine the biological impact of long-term aerosol exposure relative to exposure to conventional cigarette smoke on the A/J mouse. The assessment of the systems toxicology data is still ongoing and therefore not reported here.

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Table 7 **Overview of the main parameters investigated at/after necropsy at different time points of the study for Sham-, THS- and 3R4F- exposed female and male mice.**

Gender	Exposure	Necropsy time point (month)	Hematology, clinical chemistry, histopathology	Lung inflammation (BALF)	Lung function	Omics endpoints
female	Sham, THS, 3R4F	1	X	X	X	X
		5	X	X	X	X
		10	X			X
		18	X			X
male	Sham, THS	18	X			X

The data reported here are a compilation of the available, QA-checked data from the first 10 months of the study (clinical observations) obtained from the necropsy time points after 1, 5, and 10 months of exposure for female mice.

6.1.2.3.2.3 Results and Conclusions

Aerosol generation was reproducible throughout the 10-month exposure period, as evidenced by the stable concentrations of nicotine, TPM, CO, nicotine and selected carbonyls in the inhalation chambers, and the generated aerosols had a particle size distribution in the respirable range for rodents. Aerosols were efficiently taken up as indicated by the bio-monitoring parameters (see also Appendix A6.1.3.26). Results of carboxyhemoglobin, blood and urine nicotine and metabolites as well as metabolites of other aerosol constituents such as 3-HPMA (metabolite of acrolein) have shown that, despite some variability linked to the sample collection challenges (related to low volumes of body fluids), the mice were exposed correctly to the different aerosols. Moreover, urinary metabolites of other aerosol constituents (NNAL, SPMA, CEMA) correlated well with constituents concentrations in the aerosols as known from analytical aerosol chemistry determinations.

The test atmospheres were well tolerated by the mice as reflected by the progressive body weight gain over time. There were no remarkable acute exposure-related in-life observations with the exception of a few incidences of transient tremor occurring in mice exposed to the highest concentration of THS aerosol. During the first 10 months of the study, the mortality among the different treatment groups amounted to 81 mice out of

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1253 mice allocated to the different treatment groups (female and male mice). Of these, 6 had a technical cause, 13 were found dead and 62 were euthanized after being declared moribund. Mortality rates increased over time, and during the first 5 months of the study affected the different treatment groups evenly. At the end of month 10, a higher mortality rate was observed in THS aerosol-exposed male mice compared with Sham, whereas the mortality rates were similar in all female treatment groups. Causes of moribundity/mortality will be further investigated and reported in due course; observations included severe weight loss, enlarged hearts, large wounds, presence of tissue masses, bladder stones and distended bladders. The mortality rates observed are not different than those reported in previous studies on cigarette smoke (Stinn et al., 2013a).

The biological effects of the THS aerosol and 3R4F smoke are summarized in Table 8. In brief, exposure to 3R4F smoke induced changes in red and white blood cell profiles as well as changes in liver function parameters in the blood that were not observed in the THS aerosol-exposed A/J mice. At selected dissection time points, serum cholesterol and triglyceride concentrations were lower in 3R4F smoke- and THS aerosol-exposed mice than in Sham. However, there were no statistically significant differences in cholesterol and triglycerides concentrations between THS aerosol- and 3R4F smoke-exposed mice. The incidences of urinary glucose positivity, low urinary specific gravity and high urinary pH were higher in 24-hour urine samples collected from the THS aerosol-exposure group compared with Sham. Alterations in serum metabolic parameters as well as urinalysis data will require follow-up to distinguish the transient responses from pathologies that may develop as a result of chronic exposure. Exposure to smoke from 3R4F also resulted in higher lung weights, pulmonary inflammation, altered lung function (lower tissue elastance and resistance), and histopathological changes in the lungs that are suggestive of emphysema. No effects on these lung parameters were observed following THS aerosol exposure compared with Sham. Adaptive morphological changes in the nose and larynx were lower in THS aerosol-exposed groups compared with the 3R4F smoke-exposed mice. Nodular hyperplasia and bronchiolo-alveolar adenomas were observed in the lungs of all THS aerosol-, 3R4F smoke- and Sham-exposed animals after 10 months without clear difference between the treatments. After 10 months of exposure, minimal necrosis/degeneration was observed in the quadriceps and rectus abdominis muscles without difference between treatments. Mixed inflammatory cell infiltrates in these muscles was found to be of lower severity in the 3R4F smoke-exposed mice compared with Sham- and THS aerosol-exposed animals.

In summary, the data generated from the chronic inhalation study on mice show that exposure to aerosol from THS, produces less inflammation and adaptive changes than smoke from 3R4F, even at double the nicotine concentration in the test atmosphere. Thus the chronic toxicity of THS aerosol is much lower than that elicited by 3R4F smoke after 10 months of exposure. In addition, for a significant number of endpoints, the outcome of the exposure to THS aerosol resembles fresh air (Sham) exposure, however, a number of systemic changes require follow up at the 18-month necropsy time point.

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Table 8 Available systemic chronic toxicity, lung inflammation, lung function, and histopathology of A/J mice exposed to 3R4F smoke and THS aerosol compared with Sham-exposed mice. Data from female mice, unless indicated otherwise.

Biological Endpoints	3R4F	THSR (15020)
BODY WEIGHT DEVELOPMENT	Body weight loss during first 3 weeks of the study. At the end of 10 months of exposure, ~ 10 % lower than Sham.	Body weight loss in male and female THSR High groups during first 2 weeks. After 10 months, no difference in bodyweight relative to sham for female groups and ~ 5% lower than sham for male THSR High group.
IN-LIFE OBSERVATIONS	Trend to increased spontaneous activity and exploration behavior, more prominent at the beginning of the study.	Males: Trend to increased spontaneous activity and exploration behavior, more prominent at the beginning of the study.
RELATIVE/ABSOLUTE ORGAN WEIGHTS	Higher absolute and relative lung weights after 1, 5 and 10 months of exposure.	No difference in absolute and relative lung weights.
	Higher absolute and relative larynx and trachea weights after 5 and 10 months of exposure.	No difference in absolute and relative larynx and trachea weights.
	Higher relative heart weight after 1, 5 and 10 months (related to body weight loss).	
LUNG FUNCTION	At time points 1 and 5 months: Higher inspiratory capacity, higher compliance, lower elastance and tendency to lower resistance in single compartment model; lower tissue damping or resistance, lower tissue elastance in constant phase model; differences in quasi-static pressure volume loop and derived parameters, all indicative for emphysema.	No changes in lung function parameters.

(table continues)

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Biological Endpoints	3R4F	THSR (15020)
LUNG INFLAMMATION	Higher total BALF cell count, higher number of macrophages, dendritic cells, neutrophils, B-cells, CD4+ T cells, CD8+ T cells, and NK cells; Higher MMP activity (month 5).	In general no consistent changes in BALF cell counts. A few instances with higher cell counts without exposure concentration dependence, and when observable, much lower than 3R4F; No increase in MMP activity.
CLINICAL CHEMISTRY	Higher concentrations of total protein (months 1 and 5), albumin (months 5 and 10), bilirubin (month 5) and activity of alkaline phosphatase (months 5 and 10) in serum.	Slight serum protein, globulin, bilirubin concentration changes without exposure concentration dependence and consistency across time points. Changes much lower than those in 3R4F-exposed mice.
	Lower total cholesterol (month 5 and 10) and triglyceride (month 5) concentrations, higher glucose concentration (month 5).	Lower total cholesterol (month 5) and triglyceride (month 1 and 5) concentrations, higher glucose concentration (month 5, no concentration dependence). In general, effects similar to 3R4F at the same test atmosphere concentration.
URINALYSIS	No changes.	Glucose positivity, low specific gravity, higher pH (needs further investigation).
HEMATOLOGY	Red blood cells: higher erythrocyte counts, hematocrit, hemoglobin concentration, mean corpuscular volume and mean corpuscular hemoglobin (months 1, 5, 10) White blood cells: lower lymphocyte (months 1, 5) and higher neutrophil (month 10) counts.	Red blood cells: subtle changes, without exposure concentration dependence and consistency across time points. Changes much lower than those in 3R4F-exposed mice. White blood cells: small changes, without concentration dependence and consistency across time points.
HISTOPATHOLOGY OF THE RESPIRATORY TRACT ORGANS	<u>Nose</u>	<u>Nose</u>
	Hyperplasia and squamous epithelial metaplasia of the respiratory epithelium at nose level 1 and 2 (moderate to	Hyperplasia of the respiratory epithelium at nose level 1 (minimal to mild severity), at highest exposure concentration.

(table continues)

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Biological Endpoints	3R4F	THSR (15020)
	marked severity), atrophy of olfactory epithelium at nose level 2 (mild to moderate severity), loss of nerve bundles at level 2 (minimal to mild severity), presence of amorphous eosinophilic material in the respiratory region at nose levels 1, 2 and olfactory region nose level 4 (minimal to moderate). Findings are more pronounced after longer exposure periods.	
	<u>Larynx</u>	<u>Larynx</u>
	epithelial hyperplasia and squamous epithelial metaplasia at the base of epiglottis (severe), epithelial hyperplasia and squamous epithelial metaplasia at the floor of the larynx (severe severity), hyperplasia at the vocal cords (marked to severe severity), epithelial cornification (marked to severe severity) at base of epiglottis, ventral depression, floor of larynx and vocal cords.	epithelial hyperplasia (minimal-marked) and squamous epithelial metaplasia (normal to marked severity) at the base of epiglottis, epithelial hyperplasia (minimal to marked severity) and squamous epithelial metaplasia at the floor of the larynx (normal to marked severity), hyperplasia at the vocal cords (normal to mild severity), no epithelial cornification.
	<u>Trachea</u>	<u>Trachea</u>
	Epithelial hyperplasia (minimal to moderate severity), epithelial squamous epithelial metaplasia (normal to moderate).	No epithelial hyperplasia or squamous epithelial metaplasia.
	<u>Lung</u>	<u>Lung</u>
	Presence of unpigmented and pigmented macrophages and pigmented macrophage nests (minimal to moderate severity), presence of perivascular mononuclear cells (mild to marked severity) and neutrophils (normal to minimal severity) Alveolar emphysema at months 5 and month 10 (minimal to moderate severity) Similar incidences of nodular hyperplasia of bronchiolo-alveolar epithelium and broncho-alveolar adenomas.	No inflammatory cells, no emphysema. Similar incidences of nodular hyperplasia of bronchiolo-alveolar epithelium and broncho-alveolar adenomas.

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6.1.2.4 Overall Conclusion

The pre-clinical assessment of THS, including the regular and the menthol variants, demonstrates that the biological activity of THS aerosols is much lower than the one of 3R4F cigarette smoke. Moreover, no new hazard was identified following exposure of test systems to THS aerosol in comparison to 3R4F smoke exposure.

In vitro studies demonstrated, irrespective of the addition of menthol, a marked decrease in the biological activity of THS aerosol compared with 3R4F smoke. The cytotoxicity of GVP and TPM in the NRU assay was reduced by more than 90% compared with 3R4F smoke in both THS product variants. No genotoxicity of TPM monitored in bacterial cells (Ames assay) was observed in THSR and THSM. In the mammalian mutagenicity assay, aerosol fractions from both test and reference items showed mutagenic activity but the potency of THS was at least a factor 15 lower than 3R4F for TPM and a factor 8 lower than 3R4F for GVP.

In vivo 90-day inhalation studies on THSR and THSM aerosols demonstrated lower toxicity when compared with exposure to 3R4F smoke. Moreover, most findings in both respiratory and non-respiratory tract organs following THS aerosol exposure were considered to be associated with nicotine exposure itself as reported in previous studies (Phillips et al., 2015).

In a chronic toxicity/carcinogenicity, data collected up to after 10 months of inhalation exposure in A/J mice, has demonstrated a much lower toxicity of THS aerosol compared with smoke from the reference cigarette 3R4F. The results on the carcinogenicity endpoints are inconclusive at this point because too early; data from the 18-month necropsy will be required to evaluate the outcome.

Finally, the studies performed on THS unequivocally showed that the *in vitro* biological activity of the THSR and THSM aerosol fractions is much lower when compared with that of 3R4F smoke. Furthermore, THS aerosol exposure does not cause subchronic and chronic systemic toxicity and results in very low or no toxicity in the respiratory tract *in vivo* irrespective of the presence of menthol.

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