STUDY REPORT

90-DAY OECD INHALATION STUDY ON SPRAGUE-DAWLEY RATS TO CHARACTERIZE TOXICITY OF MAINSTREAM AEROSOL FROM THS2.2 MENTHOL STICKS AND COMPARISON TO MAINSTREAM SMOKE TOXICITY FROM 3R4F AND DDA3 1XMIS AND DDA3 2XMIS MENTHOLATED COMBUSTIBLE CIGARETTES

STUDY NUMBER 15025

PART 9 – SUMMARY OF RESULTS FOR PLUS GROUP ANIMALS

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1 Abstract

The objective of the present study was to characterize the toxicity, due to subchronic inhalation, of mainstream aerosol from the next generation product THS2.2 menthol (THS2.2M) in a 90-day inhalation study on Sprague-Dawley rats. In line with the OECD Test Guideline 413 (OECD, 2009), the mainstream aerosol from THS2.2M was generated and diluted to target nicotine concentrations of 15, 23, and 50 µg/l. The observed effects following repeated daily inhalation exposure from THS2.2M mainstream aerosol were compared to those inherent to the inhalation of mainstream smoke from the reference cigarette 3R4F (University_of_Kentucky, 2003). Additionally, DDA3 1XMIS and DDA3 2XMIS mentholated combustible cigarettes were included in the study to enable equal comparisons based on menthol content. DDA3 1XMIS and DDA3 2XMIS were designed to produce approximately 1.2 mg and 2.4 mg menthol in smoke (MIS) respectively when smoked according to ISO standard 3308 (ISO, 2012). The 3R4F, DDA3 1XMIS and DDA3 2XMIS cigarettes were only smoked to a single target nicotine concentration of 23 µg/l.

This report corresponds to an exploratory phase of the main OECD study where in-life observations of exposed animals (hereby named as the PLUS group animals) as well as the dissection of animals were performed. Animals were simultaneously exposed together with the animals belonging to the main OECD study to the respective target nicotine concentrations 6 hours a day, 5 days per week for 90 days, followed by 42 days of post-inhalation recovery period. A control group was exposed to fresh filtered air (sham). Measurement of aerosol constituents indicated lowered content of formaldehyde, acrolein, acetaldehyde and carbon monoxide in the diluted aerosol from THS2.2M as compared to reference groups. Measurement of nicotine, total particulate matter (TPM) and carbon monoxide (CO) in diluted aerosol indicated stable supply of aerosol to the experimental animals.

All aerosols were well tolerated by the animals during the exposure period. There were no differences in mortality, ocular findings and gross pathology among groups exposed to test and reference items. There were no remarkable exposure-related in-life observations. Aerosol exposure induced decreased body weight in 3R4F, DDA3 1XMIS and DDA3 2XMIS male groups compared to THS2.2M groups.

2 Introduction

See Part 1 of the report.

3 Objectives

The objective of this part of the study was to expose PLUS group Sprague-Dawley rats for 90 days to aerosol from the test item THS2.2M in Sprague-Dawley rats and to compare it to the toxicity induced by subchronic inhalation of mainstream smoke from reference 3R4F, DDA3 1XMIS and DDA3 2XMIS cigarettes. The PLUS group animals in each exposure concentration were dissected in order to provide biological samples for further exploratory studies.

4 Experimental Design and Actual Study Conditions

The PLUS animals were simultaneously exposed in the same exposure chambers as the OECD animals. Detailed description of the following points can be found in Part 1 of the report:

-Test and reference items

-Treatment administration

-Test Atmosphere Generation and Characterization

-Achieved aerosol concentrations

4.1 Test System

See Part 1 of report for details.

The first body weight measurement (study day -12, 5 days after animal arrival) showed an average body weight of 285.8 g for the males (range 223.1 g to 323.1 g) and 212.5 g for the females (range 176.6 g – 241.4 g). The rats were acclimatized for a minimum of 16 days prior to exposure to the test item.

4.2 Experimental Groups and Dosing

4.2.1 Test Groups

Male and female animals were distributed into exposure groups coded from 012 to 018 (PLUS Groups, Table 1).

4.2.2 Allocation of Animals to Treatment Groups

A total of 144 male and 168 nulliparous and non-pregnant female rats were randomly allocated simultaneously to 11 OECD groups and 7 PLUS groups by a Provantis- generated randomization sequence, based on sex and body weight. The mean body weights before allocation was 190.4 ± 11.2 g for the males, and 172.3 ± 7.4 g for the females, with a relative standard deviation (RSD) of 5.8 % in the male groups, and 4.3 % in the female groups.

After allocation, a maximum body weight RSD of 6.9 % in the male PLUS groups and 4.9 % in the female PLUS groups were obtained, both within the specification of 10%. Intergroup differences in body weight were also within 20% in accordance to OECD test guideline 413 (Table 2).

Table 1. Exposure Groups designated for exploratory 'Plus' groups.

			90d	90+42d
Group Code	**Long Name**	**Target nicotine (µg/L)**	**M/F**	**M/F**
012	Sham PLUS	0	6/6	0/6
013	3R4F PLUS	23	6/6	0/6
014	DDA3 1XMIS PLUS	23	6/6	0/0
015	DDA3 2XMIS PLUS	23	6/6	0/6
016	THS2.2M Low PLUS	15	6/6	0/0
017	THS2.2M Medium PLUS	23	6/6	0/0
018	THS2.2M High PLUS	50	6/6	0/6
	Total		42/42	0/24

Table 2. Body weights for the PLUS exposure groups following allocation to groups.

Group Name	**Sham PLUS**		**3R4F PLUS**		**DDA3 1xMIS PLUS**		**DDA3 2xMIS PLUS**	
Group Code	**012**		**013**		**014**		**015**	
Gender	Male	Female	Male	Female	Male	Female	Male	Female
Number of animals	6	12	6	12	6	6	6	12
Mean body weight (g)	191.60	172.58	190.30	172.15	190.22	172.18	190.63	172.43
SD	12.09	7.20	13.21	7.79	12.19	7.25	11.86	7.92
RSD (%)	6.3	4.2	6.9	4.5	6.4	4.2	6.2	4.6

Group Name	**THS2.2M Low PLUS**		**THS2.2M Medium PLUS**		**THS2.2M High PLUS**	
Group Code	**016**		**017**		**018**	
Gender	Male	Female	Male	Female	Male	Female
Number of animals	6	6	6	6	6	12
Mean body weight (g)	189.95	173.28	190.53	172.65	190.38	171.88
SD	11.95	8.46	11.95	8.11	12.35	8.01
RSD (%)	6.3	4.9	6.3	4.7	6.5	4.7

4.3 Procedures

General in-life condition and health of the rats were monitored, as described in Section 4.3.1. Necropsies of PLUS groups (Groups 012 to 018) were performed after the 90-day inhalation period and after 42-day post-inhalation recovery period.

4.3.1 Parameters to Monitor General Condition and Health

Parameters to monitor general condition and health (Table 3) were:

- in-life observations
- mortality/morbidity
- body weight

Table 3. General Condition and Health

Parameter	Method	Frequency	Number of Rats	Remarks
Body weight	Gravimetry	≥2 times/week during exposure	All rats	Individually
In-life Observations	Individual observations according to checklist in Provantis	Daily	Each rat systematically observed ≥4 times within the 13 weeks exposure period	Immediately after removal from exposure tube. Input data into Provantis
	Group observations according to checklist in Provantis	Daily	All rats	Immediately after removal from exposure tube. Input data into Provantis
Mortality	Observation	Daily	All rats	Immediately after removal from exposure tube. Input data into Provantis

4.3.2 Smoke Uptake and Exposure

Blood was collected for analysis of biomarkers of expsosure (BoE) in the PLUS group animals (amendment 1, deviation 3, non-GLP). BoE in blood included nicotine and cotinine in plasma as well as menthol and its metabolite p-menthane-3,8-diol (M-I) in plasma. Quantification was performed by test site 1 ABF, non-GLP.

4.3.3 Dissection and tissue collection for PLUS group animals

To avoid overlapping dissection dates with OECD groups and to harmonize dissection workflow, PLUS group animals were dissected after OECD dissection period. Dissection and tissue collection for ‘Plus’ groups 12 to 18 was performed 16 – 24 hours post exposure under non-GLP conditions. Tissues were collected for exploratory,

proteomics, transcriptomics and lipidomics analysis and further processing and analysis details will be described in separate study plans. See table 17 in the Study Plan for tissue collections after 90-day inhalation period and after 42-day post-inhalation recovery period (90+42d). Refer to the appendix 10.2 in the Study Plan for further details.

Table 4. Summary of tissues collection at 90 day after inhalation and at 90+42d from 'Plus' groups

Blood/ tissues	Planned Endpoint	Collection/ Processing	Collection	Storage
Whole blood	Transcriptomics	RNAprotect animal blood tube	RT	-80°C
Whole blood	Lipidomcs	EDTA tubes	RT	-80°C
White blood cells	Transcriptomics	CPT tubes	RT	-80°C
Serum	Proteomics	Microfuge tube	RT	-80°C
Left lung	Transcriptomics/Proteomics/	Perfuse with saline	Liq N2	-80°C
Right lung	Lipidomics	Perfuse with saline	Liq N2	-80°C
Liver	Transcriptomics/ Lipidomcis / Proteomics	Freeze in different pieces	Liq N2	-80°C
Kidneys	Transcriptomics	Freeze right and left separately	Liq N2	-80°C
Left RNE	Transcriptomics	Collection after whole body perfusion	Liq N2	-80°C
Right RNE	Proteomics	Collection after whole body perfusion	Liq N2	-80°C
Larynx and Trachea	Transcriptomics	Trimmed and rinsed with PBS	Liq N2	-80°C

Dissection and tissue collection for PLUS groups 012 to 018 took place 16 – 24 hours post exposure under non-GLP conditions. Tissues were collected for purpose of histopathological evaluation, proteomics, transcriptomics, genomics and lipidomics. See Table 4 for tissue collection list for dissection at 90 day and 90+42 day. Further processing and analysis of the collected tissues will be described in separate study reports.

Efforts to minimize RNA degradation were included by careful following of the relevant work instructions, using sterile collection tubes and petri plates that were RNase free, using surfaces and surgical tools cleaned with RNase AWAY® reagent, keeping the tissues chilled, and rapidly freezing the tissues for storage and all samples were frozen in dry ice or liquid nitrogen within 15 minutes after animal exsanguination (animal death) and stored at -80°C until shipment.

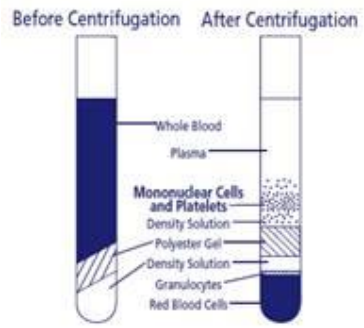
4.3.4 Tissue processing details for exploratory 'Plus' analysis (non-GLP)

4.3.4.1 Collection of whole blood and serum

Blood from rat was collected via retro-orbital puncture (WKI Exsanguinate: Retro-orbital Blood Collection). 500 µl of whole blood was collected each for RNA and DNA isolation. In addition, 500 µl of whole blood was collected for WBC count. For RNA, blood was collected in RNAprotect tube, inverted 10 times and stored 15-25°C for 2h before freezing**.** For DNA, blood was collected in EDTA tube, inverted 10 times and frozen immediately**.** For WBC count, blood was collected in EDTA tube and analysed to obtain WBC counts. For collection of serum, approximately 4 ml blood were collected in SST tube and allowed to clot at room temperature for 30 minutes. The collection tube was centrifuged according to WKI Exsanguinate: Serum and plasma preparation. To avoid modification of protein content, heat inactivation of serum was not be performed. The serum was aliquoted (125 µl aliquots) and stored at -80°C until shipment.

4.3.4.2 Collection of WBC

White blood cells were collected in CPT tubes. Blood tubes should be centrifuged within 2 hours of blood collection at room temperature 20 min at 1500 to 1800 RCF. After centrifuging, half of plasma was aspirated without disturbing the pellet and discarded. The cell layer IMMEDIATELY (figure below) in a 15mL falcon tube.



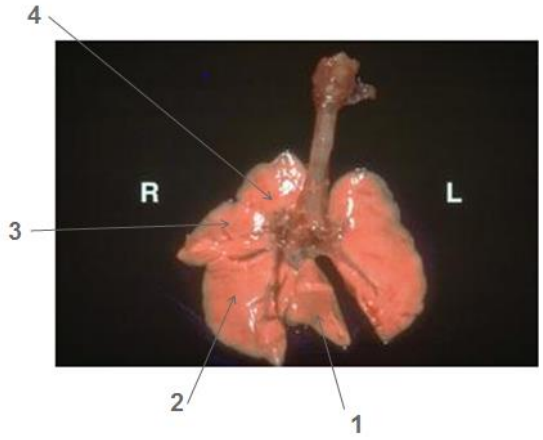
The cell layer was transferred directly into a tube containing Trizol LS reagent in a ratio of 3:1 for Trizol LS (eg. if 300 µL of cells + plasma, it will be resuspended in 900 µL of Trizol LS) and vortexed at maximum speed. Samples were immediately frozen on dry ice and stored at -80°C until shipment

4.3.4.3 Whole body perfusion

Animals were whole body perfused with cold saline to remove contaminating blood cells before removing the organs. The process was performed according to WKI Performing dissection: Lung Perfusion.

4.3.4.4 Removal and freezing of right lung for Omics

Each of the 4 lobes of the right lung was dissected and labeled separately (figure below). Each lobe was trimmed and snapped frozen in Liq N_2.



4.3.4.5 Left Lung for Omics (90d and 90+42d dissection)

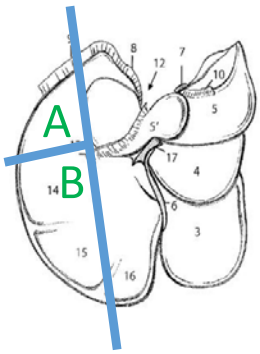
The left lung was trimmed and placed on aluminum foil and frozen in dry ice.

4.3.4.6 Collection of the non-respiratory organs

The rat's body was next halved into the upper and lower body from which the larynx, trachea, kidney and liver was collected.

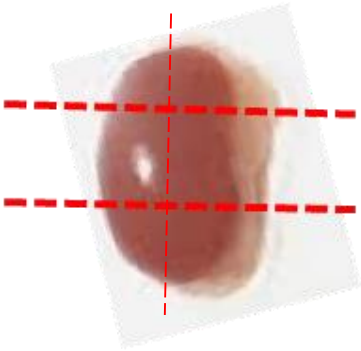
4.3.4.7 Liver

A tissue segment indicated with "A" in the figure below was divided into 2 pieces for lipidomics and RNA isolation. For RNA isolation, a tissue size approximately 3x3x3mm was excised from segment "A". Both pieces of tissues were snapped frozen in Liq N_2.The remaining portion B of the liver was snapped frozen in Liq N_2 as well.



4.3.4.8 Kidneys

The middle portion of the left and right kidney was excised (figure below). The corresponding outer portion of each kidney was snap frozen in Liq N_2.



4.3.4.9 Larynx with trachea

Larynx with trachea was collected and trimmed free from esophagus, thyroid/parathyroid and associated muscle tissue. Trachea was washed 3 times with iced cold PBS to remove blood traces before it was snap frozen in Liq N_2.

4.3.4.10 Nasal Epithelium (90d and 90+42d)

The head was excised and the lower jaw, fur, calvaria and the brain were removed from the skull. An incision sagittal to the nose was made; the nose was prised into left and right halves before removing the nasal epithelium from the exposed grooves. The left and right nasal epithelium was snap freeze in liquid nitrogen.

5 Statistical Methods

See part 1 of the report

6 Results of Biological Endpoints

6.1 General Condition and Health of the Animals- Observations

6.1.1 Group observations in PLUS groups

After the exposure, the animals showed typical signs of stress related to nose-only exposure tubes and restraint. This was manifest by post-exposure harderian gland secretion (tipically ranging between 50% to 100% of the test subjects, though typically ≥ 90% of the animals in the group presented with visible harderian secretion). Similarly, nearly all animals had wet fur after removal from the exposure tubes (≥ 75%), seemingly attributable to the accumulation of urine in the exposure tube during the exposure. Corneal and pinna reflex was observed in the majority of the animals (≥ 90%) and there were no obvious numerical differences in these observations among the groups.

6.1.2 Individual observations in OECD groups

Individual observations were performed on each animal at least 4 times during the exposure period. There were not abnormal individual observations for spontaneous activity nor for turning reflex. Reduced gripping ability was observed in 21 occasions in animals distributed across different exposure groups and distributed throughout all the exposure period.

Aerosol-exposed animals also were noted with higher incidences of wounds (on paws/legs, tail base, ear, cheeks) with 17 incidences observed in total across the exposure period being the incidence more frequent in 3R4F/DDA3 1XMIS/DDA3 2XMIS-exposed animals. Sneezing was observed in few occasions in 3R4F/DDA3 1XMIS/DDA3 2XMIS-exposed animals with no obvious numerical differences in these observations among the groups.

6.1.3 Mortality in PLUS groups

A total of 4 animals from PLUS groups (group 013 and 014) were found dead or moribund during the study (Table 5 and deviation 6). Two PLUS animals that died were replaced and there were 2 technical deaths in the PLUS groups. Animal 920093 was found dead during the week of adaptation and was replaced with a non-exposed animal according to the study plan. Eventually, later in the study this replacement animal died due to a technical death. In animal 920137, severe ulceration of the larynx was seen which may also be responsible for the moribund state of this animal because it was very pronounced. Laryngeal ulceration is considered to be a common finding after acute exposure to high concentration of smoke from reference cigarettes. Animals 920014, and 920139 died because of technical reason.

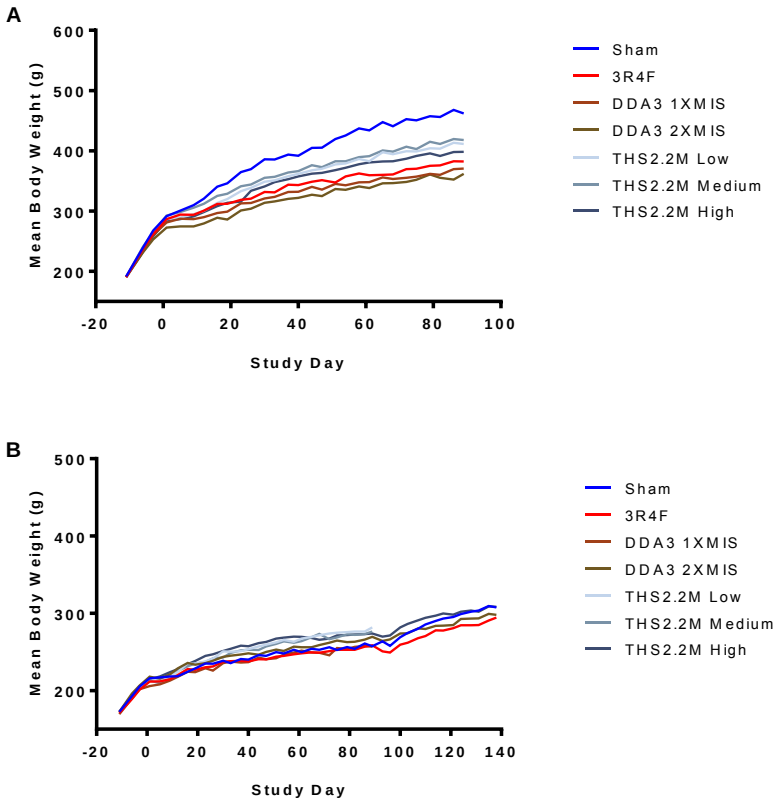
Table 5. Animal mortality in OECD groups during the experimental phase
CAN: coded animal number; UAN: uncoded animal number.

Date	GROUP	UAN	CAN	Replacement animal	CAN
1-Jun-14	3R4F PLUS	0132001	920014	9992004	920093
30-Jun-14	3R4F PLUS	0132001	920014	Not replaced	NA
30-Jun-14	3R4F PLUS	0132002	920139	Not replaced	NA
30-Jun-14	DDA3 1XMIS PLUS	0142001	920115	8832001	920137

6.2 Body Weight

The body weights trends before exposure, during first week of adaptation and progression during 90 day exposure and post-exposure periods were very similar to that of OECD groups with all animals gaining body weight over time throughout the 90-day exposure period. Only female were included in the recovery groups, the trends showed a more rapid body weight gain in the 42-day post-inhalation recovery period as seen in the OECD groups.

Figure 1. Mean Body weight, male rats (A) and female rats (B) from PLUS groups



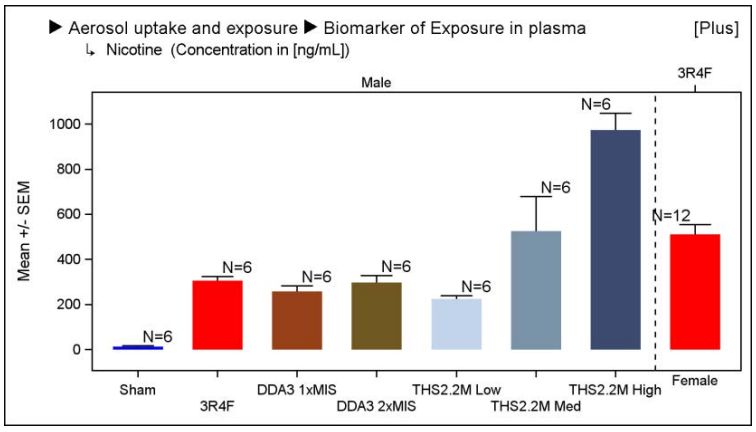
6.3 Smoke uptake

6.3.1 Biomarkers of exposure in blood

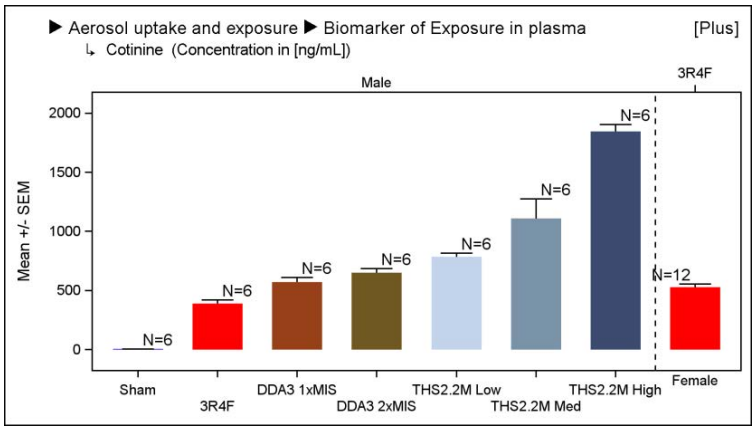
Biomarkers of exposure in blood included nicotine and cotinine in plasma as well as menthol and its metabolite p-menthane-3,8-diol (M-I) in plasma. The plasma levels of nicotine were approximately 2 to 3-fold higher in the THS2.2M high group animals compared to 3R4F, DDA3 1XMIS and DDA3 2XMIS-exposed rats (Figure 2). Cotinine levels were approximately 2.8 to 4.7-fold higher in the THS2.2M high group animals compared to 3R4F, DDA3 1XMIS and DDA3 2XMIS-exposed rats. Observed nicotine concentrations correlated well to the amount of nicotine present in the aerosols that were delivered to the exposed rats.

Figure 2. Mean concentration of nicotine and cotinine present in the blood of male and female rats (separated by dotted line) during the 90-day exposure period.

A) Mean nicotine concentration (ng/mL) in male and female rats.



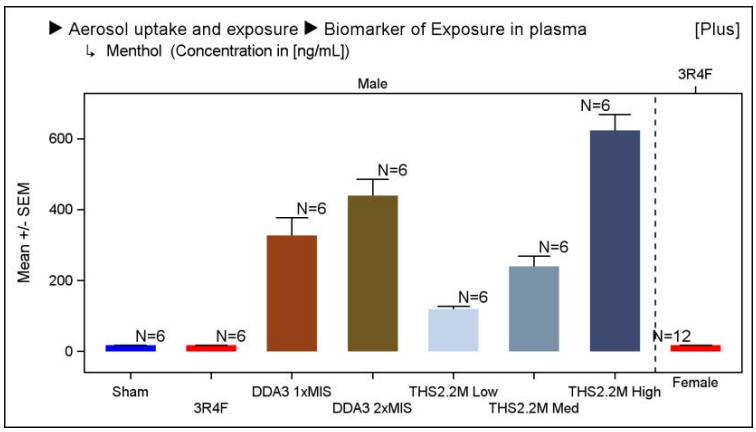
B) Mean cotinine concentration (ng/mL) in male and female rats.



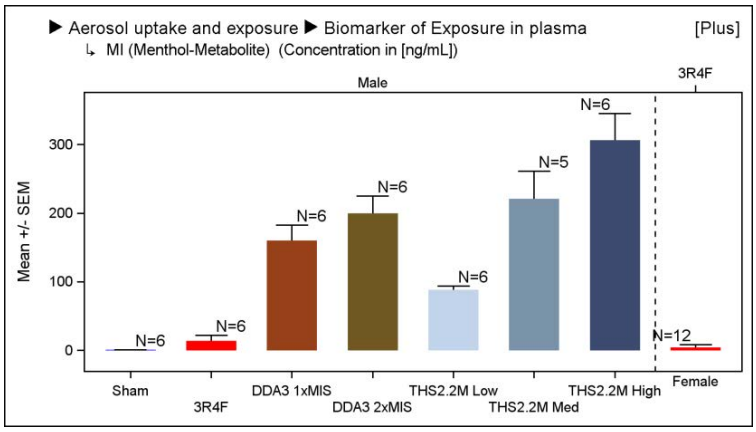
The uptake of menthol was verified by quantification of menthol and menthol metabolite, ρ-methane-3,8-diol in plasma (Figure 3). The plasma concentrations of menthol and ρ-methane-3,8-diol in the THS2.2M exposed rats were approximately 1.4-fold and 1.5-flod that of DDA3 2XMIS exposed rats respectively. The observed plasma menthol and M-I concentrations showed a good concentration-dependent response in the THS2.2M-exposed and DDA3 1XMIS/DDA3 2XMIS-exposed rats.

Figure 3. Mean concentration of menthol and ρ-methane-3,8-diol (M-I) present in the blood of male (left panel) and female rats (separated by dotted line) during the 90-day exposure period.

A) Mean concentration of menthol (ng/mL) in male and female rats.



B) Mean concentration of M-I (ng/mL) in male and female rats.



6.4 Dissection and tissue collection for PLUS group animals

Organs from all PLUS group animals with the exception of moribund and unscheduled death animals were successfully harvested and shipped out for further analysis.

7 Discussion

The objective of this 90-day inhalation study was to determine whether subchronic inhalation of THS2.2M aerosol induced toxicity in Sprague-Dawley rats and to compare it to the toxicity induced by subchronic inhalation of MS from the reference cigarettes 3R4F and combustible mentholated cigarettes (DDA3 1XMIS and DDA3 2XMIS). In-life observations during the exposure phase were conducted in accordance to GLP guidelines while the dissection of PLUS group animals was conducted under non-GLP conditions. Male and female Sprague-Dawley rats were exposed for 90 days, 5 days per week, 6 hours per day. The recovery animals were exposed for 90 days before the 42 days of post-inhalation recovery period. The target aerosol concentration of THS2.2M test item was 15 µg/L, 23 µg/L and 50 µg/L nicotine. The target aerosol concentration of 3R4F, DDA3 1XMIS and DDA3 2XMIS reference items was 23 µg/L nicotine. In part 1 of the report it is described that frequent monitoring of nicotine concentrations at the breathing zone of the rats in the exposure chambers demonstrated that there was a reproducible and stable nicotine concentration throughout the 90-day exposure period and that the average nicotine concentrations were within specified range (± 10% of the target concentration) in all chambers. There were only 3 incidences where daily average nicotine concentration in group 3R4F was slightly out of the daily specification of nicotine target concentration (deviation 5). However, these slight variations were minimal and final target nicotine concentration was within the specified range. The stability of aerosol produced was also reflected by the small variability in the daily nicotine, TPM and CO concentrations in the test atmospheres. The particle size distribution was consistent with that obtained from previous studies and indicated that particles were compatible with inhalation and equally respirable among all aerosol-exposed groups.

In-life observations, mortality and body weight development of PLUS group animals were similar and consistent with that of OECD group animals. There were no differences in mortality rate and gross pathology observations between the 3R4F/ DDA3 1XMIS/ DDA3 2XMIS-exposed and THS2.2M-exposed groups. Reduced weight gain was observed in male 3R4F groups and THS2.2M groups. Decreased body weight development was observed in male 3R4F/ DDA3 1XMIS/ DDA3 2XMIS groups and THS2.2M groups. The body weight effect in the male rats exposed to reference aerosols was greater than exposure to THS2.2M aerosol and is likely due to effects of harmful smoke constituents such as acrolein present in the reference aerosols as previously described (Feron et al., 1978). Due to the very low levels of acrolein in the THS2.2M groups, the body weight effects observed in the male rats exposed to THS2.2M aerosol are probably driven by nicotine (Chowdhury, 1990). For both male and female rats, the body weight gain in 3R4F, DDA3 1XMIS and DDA3 2XMIS groups was lower than THS2.2M groups likely due to lower toxicity from THS2.2M aerosol.

8 Conclusion

This study evaluated the biological effects of inhaled aerosol from THS2.2M in comparison with the 3R4F, DDA3 1XMIS and DDA3 2XMIS reference cigarettes. The 90-day inhalation study was performed according to the OECD Test Guideline 413. Aerosol generation was reproducible and the aerosol was demonstrated to be efficiently taken up by the PLUS group animals as indicated by the biomonitoring parameters in blood. No additional remarkable clinical (in-life) observations were observed in THS2.2M-exposed PLUS group animals compared with 3R4F/ DDA3 1XMIS / DDA3 2XMIS-exposed PLUS group animals. In-life observations, mortality and body weight development were consistent with OECD group animals and lower toxicity induced by THS2.2M test item as compared to conventional cigarettes was evident from higher body weight gained over time.

9 References

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10 Abbreviations and Definition of Terms

Abbreviation/Term	
AP	alkaline phosphatase
ALT	alanine aminotransferase
AST	aspartate aminotransferase
BALF	bronchioalveolar lavage fluid
CO	carbon monoxide
EDTA	ethylenediamine tetraacetic acid
EGAFS	ethanol glycerol acetic acid formaldehyde solution
FLC	free lung cell
FTC	Federal Trade Commission (USA)
GLP	Good Laboratory Practice
GSD	geometric standard deviation
HbCO	carboxyhaemoglobin
HB	haemoglobin
HCT	haematocrit
HEPA	high efficiency particulate air
ISO	International Organization for Standardization
KMIS	Knowledge Management Information Services
LIMS	Laboratory Information Management System
IU	international units
LLOQ	lower limit of quantification
LPT	Laboratory of Pharmacology and Toxicology
MMAD	mass mean aerodynamic diameter
MRS	mean relative to sham
MS	mainstream smoke

Abbreviation/Term	
N	number of individual values (or determinations etc.)
NA	not applicable
NACLAR	National Advisory Committee for Laboratory Animal Research
NADH	nicotinamide adenine dinucleotide
NTP	National Toxicology Program
OECD	Organization for Economic Co-operation and Development
p	probability of receiving the value of the test statistic or a more extreme value of the test statistic, if the null hypothesis holds
PLT	platelet(s)
PMIRL	Philip Morris International Research Laboratories
PMRL	Philip Morris Research Laboratories
QL	quantification limit
RSD	relative standard deviation
SD	standard deviation
SE(M)	standard error (of the mean)
SOP	standard operating procedure
SPF	specific pathogen-free
TPM	total particulate matter

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