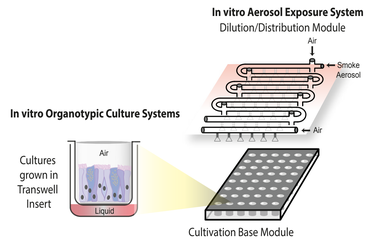
In Vitro Systems Toxicology Assessment of Exposure to Aerosol from a Carbon Heated Tobacco Product as Compared with Exposure to Cigarette Smoke: The Impact on Nasal and Small Airway Epithelial Cultures

Anita R. Iskandar, Yannick Martinez, Carole Mathis, Patrice Leroy, Florian Martin, Alan Sewer, Laura Ortega Torres, Shoaib Majeed, Keyur Trivedi, Stefan Frentzel, Emmanuel Guedj, Celine Merg, Ashraf Elamin, Nikolai V. Ivanov, Manuel C. Peitsch, Julia Hoeng
Philip Morris International R&D, Philip Morris Products S.A., Neuchâtel, Switzerland (part of Philip Morris International group of companies)

Introduction

Toxicological assessment of tobacco products should provide relevant indication of the health risk for humans. Advances in tissue engineering have allowed the development of in vitro organotypic cultures with an air-liquid interface, thus permitting a direct exposure to inhaled chemicals. Using human organotypic nasal and small airway epithelial cultures, this study assessed the impact of an aerosol from a carbon heated tobacco product (CHTP) 1.2—a candidate modified-risk tobacco product (MRTP)*—compared with cigarette smoke (CS) at similar nicotine levels. Various endpoints including cytotoxicity, histology, ciliary beating function, cytochrome P450 1A1/1B1 activity, and secreted pro-inflammatory mediators, were complemented by a systems biology analysis of the transcriptomes to assess the exposure impact at different post-exposure time points. The overall data demonstrate a substantially reduced biological impact of CHTP1.2 aerosol exposure compared with CS in both nasal and small airway cultures.

Materials & Methods



Nasal

Concentration of Nic (mg/L)	
3R4F	CHTP1.2
0.00 (Air)	0.00 (Air)
0.15	0.14
0.27	0.27
-	0.54

Small Airway

Concentration of Nic (mg/L)	
3R4F	CHTP1.2
0.00 (Air)	0.00 (Air)
0.16	0.15
0.26	0.30
-	0.40

Exposure Characterization:
Nicotine concentrations in smoke/aerosol

Biological Endpoint:	Before	After	Post-Exposure Measurements 4 h	24 h	48 h	72 h
Culture histology					x	x
Cytotoxicity			x	x	x	x
Secreted mediators				x	x	x
Transcriptomics			x	x	x	x
Ciliary beating analysis	x	x		x	x	x

The impact of 28 min exposure to CS (from 3R4F reference cigarette, University of Kentucky) and to aerosol (from CHTP1.2, Philip Morris International R&D) were assessed using human organotypic nasal cultures (reconstituted from the nasal epithelial cells of a 41 year-old female, nonsmoker donor) and small airway cultures (reconstituted from the small airway epithelial cells of a 55 year-old female, nonsmoker donor). A paired design was implemented so that in parallel to the exposure to CS or to CHTP1.2 aerosol, the cultures were also exposed in the same exposure module and during the same exposure run to air. For each of the culture models, a series of experimental repetitions was conducted to increase the assessment robustness (N = 3 exposure runs/repetition).

Characterization of the 3R4F smoke and CHTP1.2 aerosol in the exposure system (Vitrocell 24/48®) includes the measurements of nicotine in the diluted 3R4F smoke and THS2.2 aerosol throughout the studies. The diluted smoke/aerosol was trapped in EXtrelut 3NT® column and subjected to gas chromatography-flame ionization detection to determine the nicotine concentrations at the specific dilutions within each experimental week.

Cytotoxicity was assessed by measuring the adenylate kinase activity in the basolateral medium of the cultures using ToxiLight™ bioassay kit (Lonza, Rockland, MA, USA), according to the manufacturer's instructions.

For the histological analysis, the cross-sections of the organotypic epithelium cultures were analysed after hematoxylin and eosin and alcian blue staining.

Concentrations of pro-inflammatory mediators were measured from the basolateral medium of the exposed cultures using Luminex® xMAP® technology and commercially available assay panels (EMD Millipore Corp) according to the manufacturer's instructions. The culture media were collected at different post-exposure time points.

mRNA microarrays were done using 100 ng of total RNA (per sample) that were reverse-transcribed and amplified to cRNA using the Affymetrix® HT 3' IVT PLUS kit. Causal network enrichment analysis was done using the Network Perturbation Amplitude (NPA) methodology (Hoeng et al., 2014; Martin et al., 2012; Martin et al., 2014) was used to contextualize high dimensional transcriptomics data by combining gene expression ($\log_2$-fold-changes into fewer differential node values (one value for each node of a causal biological network model). The collection of causal biological networks used in the study(s) was the human network suite CBN v1.3 (Boué et al., 2015).

References

Family Smoking Prevention and Tobacco Control Act, 2009. The term modified risk tobacco product (MRTP) is defined by the US Family Smoking Prevention and Tobacco Control Act (FSPTCA, 2009), and is to be understood as a general term which covers any product option, including e-cigarettes, electronic nicotine delivery systems (ENDS), and potential reduced exposure products (PREPs). The term "modified risk tobacco product" means any tobacco product that is sold or distributed for use to reduce harm or risk of tobacco-related diseases associated with commercially marketed tobacco products. ***BOUE, S., et al. 2015.*** Causal biological network database: a comprehensive platform of causal biological network models focused on the pulmonary and vascular systems. Database, 2015, bav030. ***HOENG, J., et al. 2014.*** Case study: the role of mechanistic network models in systems toxicology. Drug discovery today, 19, 183-192. ***MARTIN, F., et al. 2014***. Quantification of biological network perturbations for mechanistic insight and diagnostics using two-layer causal models. BMC Bioinformatics, 15, 238. ***MARTIN, F., et al. 2012***. Assessment of network perturbation amplitudes by applying high-throughput data to causal biological networks. BMC systems biology, 6, 54.

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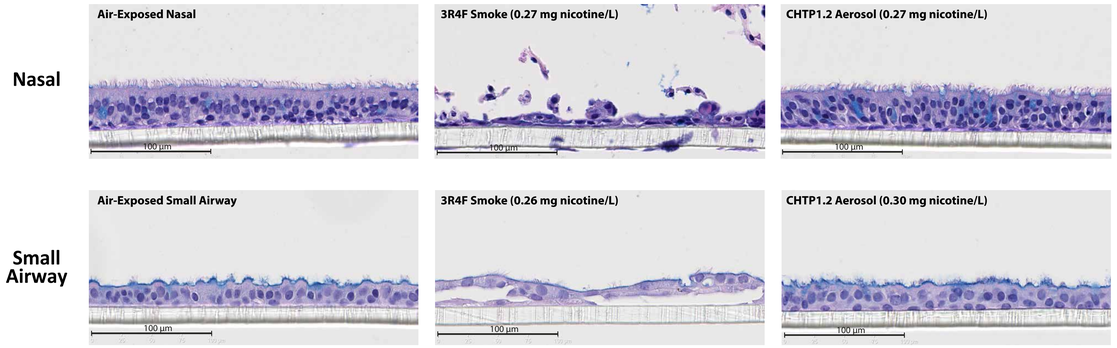
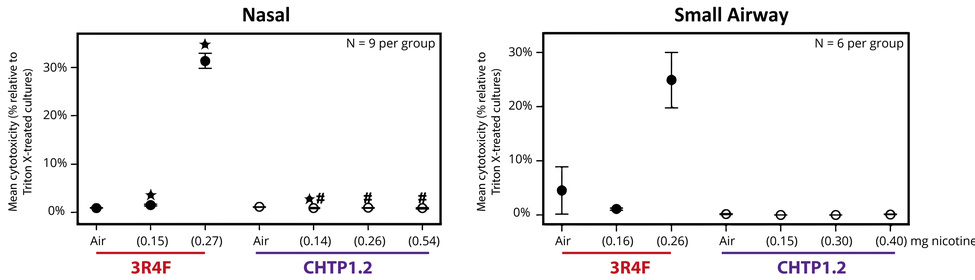
Results

Culture Histology 72 h After Exposure



Hematoxylin-Eosin/Alcian Blue staining was performed in cultures 72 h post-exposure to 3R4F smoke or CHTP1.2 aerosol. At comparable nicotine concentration (0.27 - 0.30 mg/L), damage to the epithelial layer was observed in cultures exposed to 3R4F smoke but not in those exposed to CHTP1.2 aerosol.

Cytotoxicity 72 h Post-Exposure



Cytotoxicity levels were determined based on the levels of adenylate kinase released into the basolateral media following exposure. Upon cellular damage, adenylate kinase is released from the intracellular to the extracellular compartment. The level of cytotoxicity was reported relative to the Triton X-treated cultures (considered as 100% cytotoxicity). Greater cytotoxicity levels were observed following exposure to the higher concentration of 3R4F smoke. Less cytotoxicity levels were observed following exposure to all concentrations of CHTP1.2 aerosol. ★p-value < 0.05 vs. its corresponding air control. #p-values 0.05 between CHTP1.2 and 3R4F at comparable concentrations.

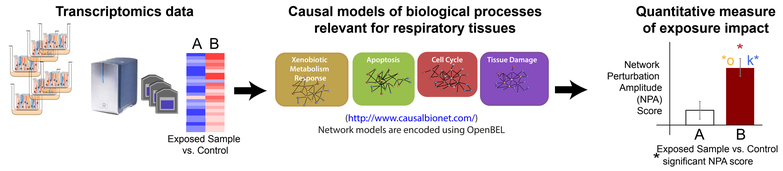
Secretion of Pro-inflammatory Mediators Following Exposure

	24 h Post-Exposure						48 h Post-Exposure						72 h Post-Exposure						
	Air	(0.15)	Air	(0.14)	(0.26)	(0.54)	Air	(0.15)	Air	(0.14)	(0.26)	(0.54)	Air	(0.15)	Air	(0.14)	(0.26)	(0.54)	
	3R4F		CHTP1.2				3R4F		CHTP1.2				3R4F		CHTP1.2				
Nasal	4672	6036	3682	4670	4642	5049	9075	13614	9710	7595	16543	11456	9886	16286	9892	10732	8526	11229	CXCL1
	1546	4604	783	995	1149	1685	1607	7749	1484	1886	2648	3577	2373	6357	1988	2445	2049	2898	CXCL8
	22	34	20	23	23	25	66	104	51	65	62	83	82	100	59	70	48	71	CCL20
	1526	2921	976	1242	1134	1018	5369	4974	2742	4108	3380	3886	6013	5704	2568	3922	2754	3794	MMP-1
	5549	8211	3412	7411	8795	7240	17502	30196	5962	20334	19501	21351	26420	33184	13779	16886	16461	16918	MMP-9
	5479	4297	3685	2763	2929	4466	9832	14096	22455	17650	19003	19235	17511	34076	19291	15565	11313	18974	TIMP1
	91	290	98	144	175	259	183	549	264	206	250	341	332	888	444	355	424	705	VEGFA
Small Airway	8237	7488	7241	7611	10980	7327	22731	21719	24801	24450	20722	19388	46986	120539	27699	30953	25398	24390	CXCL1
	2655	8852	2436	2584	6435	5094	6926	17823	7386	7819	8065	8629	20287	63860	8939	9404	10659	9778	CXCL8
	23	73	33	21	59	42	84	159	84	94	60	86	93	224	98	89	77	58	CCL20
	876	11211	691	659	2655	2822	2218	19652	1997	2421	3171	5223	18515	32736	4055	3783	5773	5856	MMP-1
	3639	11202	2968	1982	12027	6495	12751	31834	9571	10925	12791	20359	52706	72830	17933	13620	25574	23352	MMP-9
	2737	6720	2625	2955	3487	3923	4804	23934	4109	4820	6104	7117	5622	54829	6161	5861	7124	9062	TIMP1
	92	239	90	95	117	155	232	828	213	241	343	294	426	686	349	324	347	443	VEGFA
	Air	(0.16)	Air	(0.15)	(0.30)	(0.40)	Air	(0.16)	Air	(0.15)	(0.30)	(0.40)	Air	(0.16)	Air	(0.15)	(0.30)	(0.40)	
	3R4F		CHTP1.2				3R4F		CHTP1.2				3R4F		CHTP1.2				

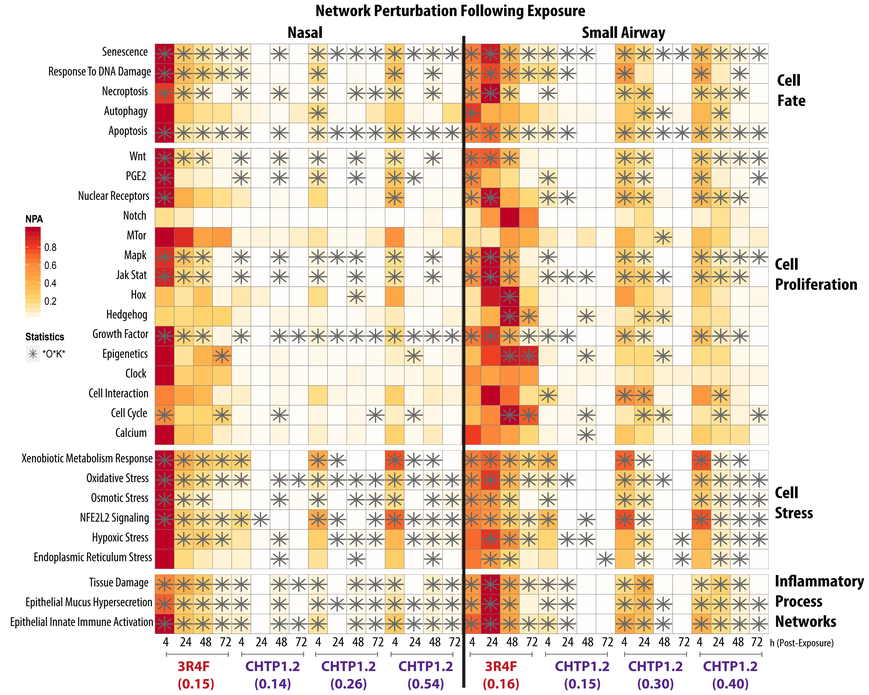
Lowest to the highest concentration (mean, pg/mL) per mediator (row)

Various mediator concentrations secreted from the cells were measured to assess the inflammatory response of the cultures following 3R4F smoke and CHTP1.2 aerosol exposure. Numbers indicate the mean concentrations of pro-inflammatory mediator secreted into the basolateral media following exposure to 3R4F smoke and CHTP1.2 aerosol (N = 9 per group for nasal; N = 6 for small airway). Only the cultures exposed to the smoke or aerosol at sub-toxic levels were included, i.e., the samples exposed to the higher dose of 3R4F smoke were excluded. Relative to the air-exposed samples, the concentrations of the secreted mediators were markedly increased following 3R4F smoke exposure. In general, smaller alterations in the mediator concentrations were observed following CHTP1.2 aerosol at all doses tested, even at a dose where the nicotine concentrations were more than twice of the nicotine concentrations in 3R4F smoke.

Perturbation of Biological Pathways and Processes Following Exposure: Network-based Analysis of Transcriptome Profiles

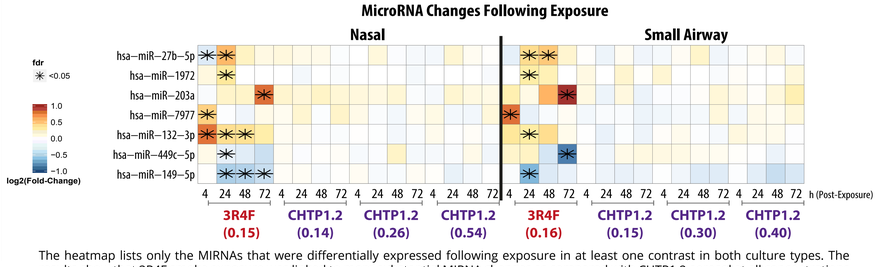


Network Perturbation Following Exposure



A network-based systems biology approach was conducted using transcriptomics data from cultures exposed to the smoke or aerosol at sub-toxic levels, i.e., the samples exposed to the higher dose of 3R4F smoke were excluded. This approach uses biological network models that contain a series of cause-and-effect relationships (Boué et al., 2015). The network models can be grouped into network families, representing more general biological processes (e.g., Cell Proliferation, Cell Fate, Cell Stress, or Inflammatory Process Networks network). CHTP1.2 aerosol-induced network perturbations were lower than those following 3R4F smoke (N = 9 per group in nasal; N = 6 per group in small airway studies). NPA, network perturbation amplitude.

MicroRNA Changes Following Exposure



The heatmap lists only the MIRNAs that were differentially expressed following exposure in at least one contrast in both culture types. The results show that 3R4F smoke exposure was linked to more substantial MIRNA changes as compared with CHTP1.2 aerosol at all concentrations tested (N = 9 per group in nasal; N = 6 per group in small airway studies).

Conclusions

Collectively, the results show that the impact of CHTP1.2 aerosol on nasal epithelium was considerably lower than 3R4F smoke at similar nicotine concentrations, in regard to:

- Cytotoxicity levels (based on the levels of adenylate kinase released into the basolateral media) and culture morphology at 72 h post-exposure
- Inflammatory responses (based on the secreated pro-inflammatory mediator levels) following exposure
- Perturbation of biological processes (modeled in the causal network models) derived from the global gene expression and MIRNA changes following exposure.

Using this systems toxicology approach, biological processes and/or signaling pathways could be identified from the global gene expression changes that were not captured by the classical functional measures (culture morphology, cytotoxicity, and secretion of pro-inflammatory mediators). As compared with the biological impact of 3R4F smoke, the aerosol from the candidate modified-risk tobacco product CHTP1.2 elicited much lower impact in all measured endpoints in the human nasal and small airway cultures, even when the nicotine concentrations in the CHTP1.2 aerosols were 2-3 times higher than that in 3R4F smoke.

Competing Financial Interest
The research described in this poster was sponsored by Philip Morris Products SA