In vitro evaluation of the biological impact of Harmful and Potentially Harmful Constituents of tobacco smoke using a systems toxicology approach

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Introduction

Cigarette smoke (CS) causes many serious diseases including chronic obstructive pulmonary disease and cancer. CS is a complex aerosol containing over 6,000 chemicals (Rodgman and Perfetti 2013). Thus, it is difficult to determine the contribution of individual smoke constituents to overall toxicity, as well as the molecular mechanisms by which they exert their effects when the smoke is inhaled.

The individual impact of 14 selected Harmful and Potentially Harmful Constituents (HPHC) (FDA HPHC 2012) was investigated by establishing a High Content Screening (HCS) method in normal human bronchial epithelial (NHBE) cells. Thirteen multi-parametric indicators of cellular toxicity were measured using the HCS platform. In addition, the study was complemented with a microarray-based transcriptome analysis followed by a computational approach leveraging mechanistic network models to identify and quantify perturbed molecular pathways. HPHCs were evaluated in a wide range of concentrations and at different time points.

HCS-based analysis showed positive results only at the highest (toxic) doses tested. Toxicity mechanism(s) varied with the different HPHCs and included: DNA damage/growth arrest, activation of cellular stress responses, oxidative stress, mitochondrial stress and apoptosis / necrosis. Based on the effective doses and the number of mechanisms activated, the most hazardous constituents tested were formaldehyde, catechol, and acrolein. A microarray-based systems toxicology approach was used in these three constituents to further investigate molecular mechanisms of toxicity. At the highest (toxic) dose, the results are consistent with and complement HCS findings. Toxic doses were selected based on literature review. In addition, we provide mechanistic insight on the biological perturbations caused by formaldehyde, catechol, and acrolein at doses equivalent to single exposure to conventional cigarettes (CC) and modified-risk tobacco products (MRTPs).

Materials and Methods

Selection of HPHCs

14 constituents were selected from a list of 93 HPHCs in tobacco products and tobacco smoke established by the U.S. Food and Drug Administration [2]. (**Table 1**).

- O-toluidine	- Nicotine	- 4-Aminobiphenyl	- **Acrolein**
- Styrene	- 2-Naphthylamine	- NNN	- **Catechol**
- Cadmium	- 1,3 Butadiene	- NNK	- **Formaldehyde**
- Acrylonitrile	- Propionaldehyde		

Table 1. List of selected HPHCs. In blue, constituents selected for transcriptomics

High Content Screening

13 standard toxicological endpoints were established in normal human bronchial epithelial (NHBE) cells (**Table 2**). Cells were seeded onto 96-well plates for 24h hours and then exposed, in triplicate, to a wide range of single HPHC concentrations. Cells were exposed for 4h, 8h and 24h.

- Cell loss	- Membrane permeability	- Phospho-H3	- Cytochrome C release
- DNA Structure	- Mitochondrial mass	- Phospho-H2AX	- DHE
- Nuclear size	- Mitochondrial potential	- Phospho-cJun	- GSH content
			- Caspase 3/7

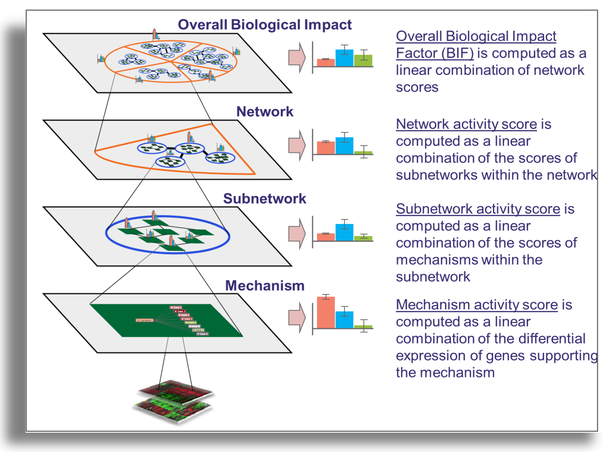
Table 2. List of HCS endpoints

Based on the number of positive HCS endpoints (**Table 3**), we selected acrolein, catechol and formaldehyde for further analysis using a transcriptome-based systems biology approach. NHBE cells were exposed in triplicate to each single HPHC at three doses : High (toxic), medium (CC) and low (MRTP). Cells were exposed for 4, 8 and 24h.

	HCS (High dose)								
	Acrolein			Catechol			Formaldehyde		
	4h	8h	24h	4h	8h	24h	4h	8h	24h
GSH depletion	✓	✓	✓	X	X	X	X	X	✓
pH3 (mitosis)	✓	✓	✓	✓	X	X	X	X	X
DNA structure	X	X	✓	X	✓	✓	✓	X	✓
Cell loss	X	X	✓	✓	✓	✓	X	X	✓
pH2AX (DNA damage)	X	X	X	X	X	✓	✓	✓	X
Membrane permeability	X	X	X	X	X	✓	X	X	✓
Mitochondrial potential	X	X	X	X	X	X	X	X	✓
Mitochondrial mass (loss)	X	X	X	X	X	X	X	X	✓
Cytochrome C release	X	X	X	X	X	X	X	X	✓
cJun (Cell stress)	X	X	X	X	X	X	X	✓	X

Table 3. Summary of HCS results for acrolein, catechol and formaldehyde.

Quantifying process activity and biological impact factor



In order to more accurately characterize the molecular mechanisms perturbed upon HPHC exposure, we used a system-level approach called Network Perturbation Amplitude (NPA) scoring. NPA computes the amplitudes of treatment-induced perturbations by applying the transcriptomic data to a set of network models that describe a range of key biological processes (cellular stress inflammation, proliferation, DNA damage apoptosis, necroptosis, autophagy and senescence). NPA scoring enables identification of activated molecular mechanisms, and an assessment of how these molecular mechanisms act together within different biological processes. The combination of all network scores is then used to calculate and overall Biological Impact factor (BIF).

Conclusion

This study shows that the combination of standard toxicological endpoints with a systems-based impact assessment allows for a more robust scientific basis for toxicological assessment of smoke constituents, while gaining insight on the molecular mechanisms activated upon exposure. We have established an *in vitro* Systems Toxicology platform which will now be applied to a broader selection of HPHCs and their mixtures. This study may be used to support the approach to develop a systems biology-based risk assessment for Modified Risk Tobacco Products (MRTPs).

References

1. Rodgman A, Perfetti TA (2013): The chemical components of tobacco and tobacco smoke. 2nd edition; CRC Press, Taylor & Francis Group: Boca Raton.

2. FDA HPHC (2012): Harmful and Potentially Harmful Constituents in Tobacco Products and Tobacco Smoke; Established List. U.S. Department of Health and Human Services, Food and Drug Administration, Center for Tobacco Products. Rockville, MD. In. Federal Register 77, April 3, 2012. pp 20034-20037.

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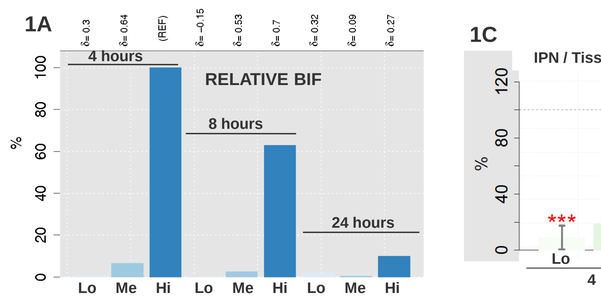
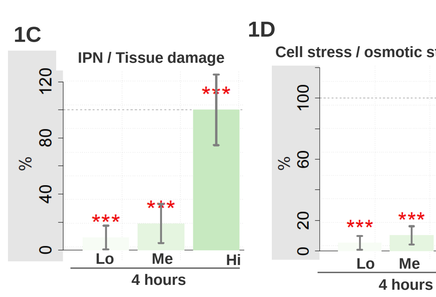
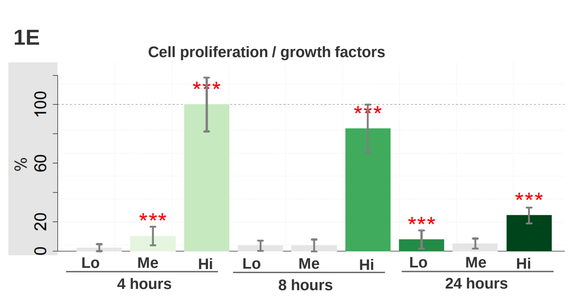
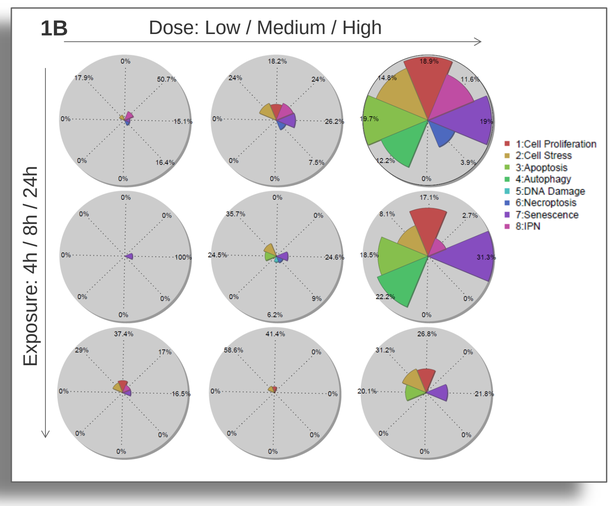
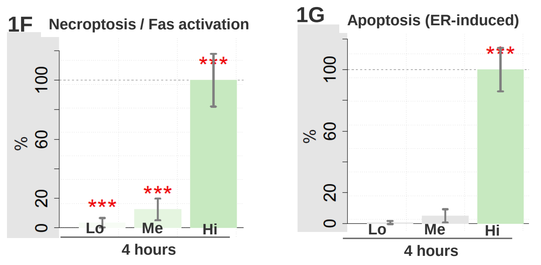
Results: ACROLEIN











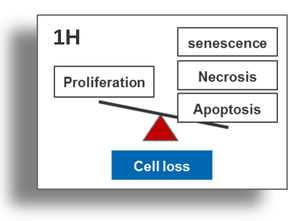
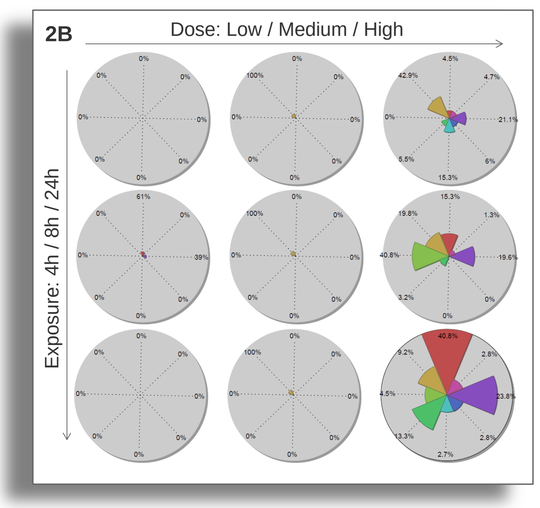
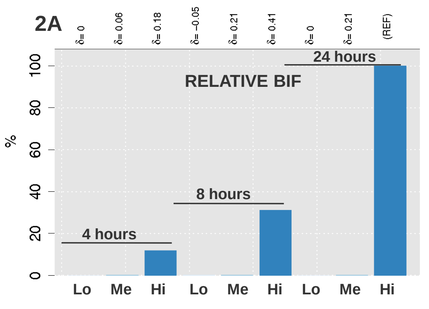
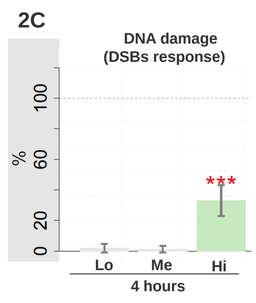


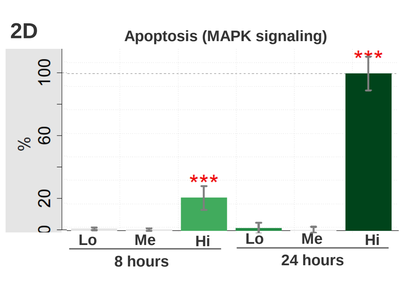
Figure 1. Biological Impact Factor (BIF) for NHBE cells exposed to Acrolein. 1A) Overall BIF for NHBE cells exposed to a single dose equivalent to a pMRTP (Lo: 0.2µM), to a single dose equivalent to CC (Me: 2.7µM) and single toxic dose (Hi: 50µM) of acrolein. Barplot shows BIF values relative to the maximum response group (REF). The δ values (-1 to 1) indicate how similar the underlying network perturbations are with respect to the REF. Scores were computed using transcriptomic profiling data of treated cells compared to untreated. 1B) Starplots show the decomposition of the overall BIF into its main mechanistic components for all doses and timepoints. The labels in each plot show the contribution (in percentage) of each network to the overall effect. They all sum 100%. 1C-G) NPA calculations for cell-type specific subnetworks at different doses and time points. Error bars represent 95% confidence intervals. *** indicates significant value. 1H) Exposure to acrolein results in pro-apoptotic and pro-survival signals. 1H) The balance between signals determines ultimately cell fate.

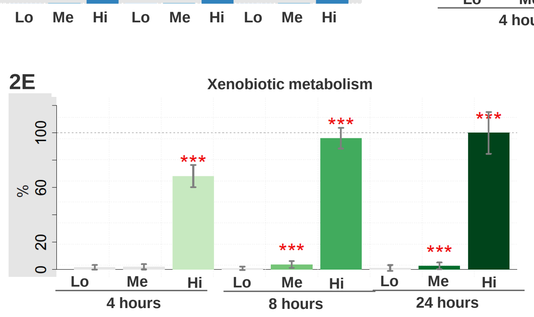
Results: CATECHOL

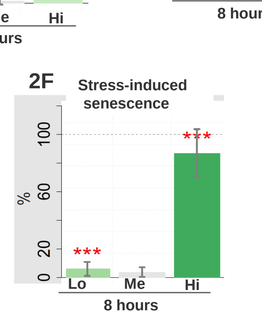












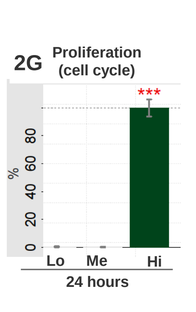
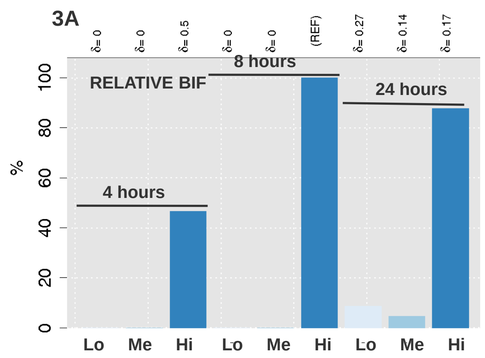
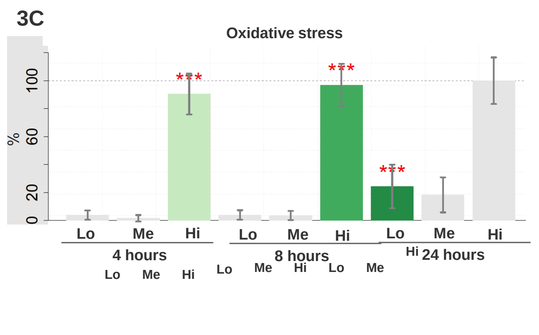
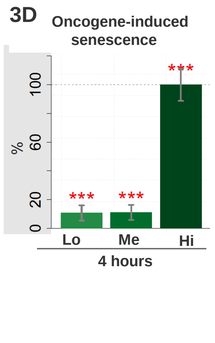


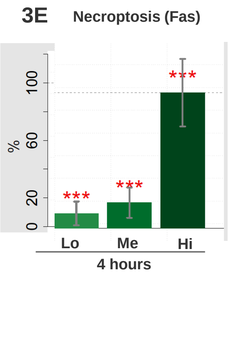
Figure 2. Biological Impact Factor (BIF) for NHBE cells exposed to Catechol. 2A) Overall BIF for NHBE cells exposed to a single dose equivalent to a pMRTP (Lo: 0.2µM), to a single dose equivalent to CC (Me: 0.9µM) and single toxic dose (Hi: 30µM) of catechol. Barplot shows BIF values relative to the maximum response group (REF). 2B) Starplots show main mechanistic components for all doses and timepoints. The labels in each plot show the contribution (in percentage) of each network to the overall effect. They all sum 100%. 2C-G) NPA calculations for cell-type specific subnetworks. Error bars represent 95% confidence intervals. *** indicates significant value. At high doses, catechol induces oxidative stress, leading to DNA damage, senescence , apoptosis and cell cycle arrest (consistent with HCS results) At medium doses, catechol induces cellular stress and activates the xenobiotic metabolism response . This response is not activated at low doses.

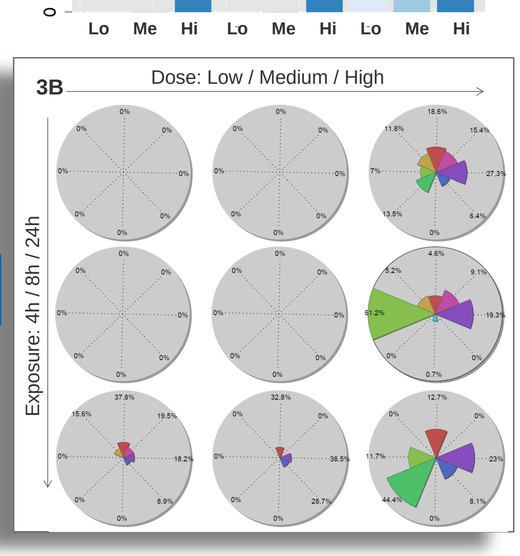
Results: FORMALDEHYDE

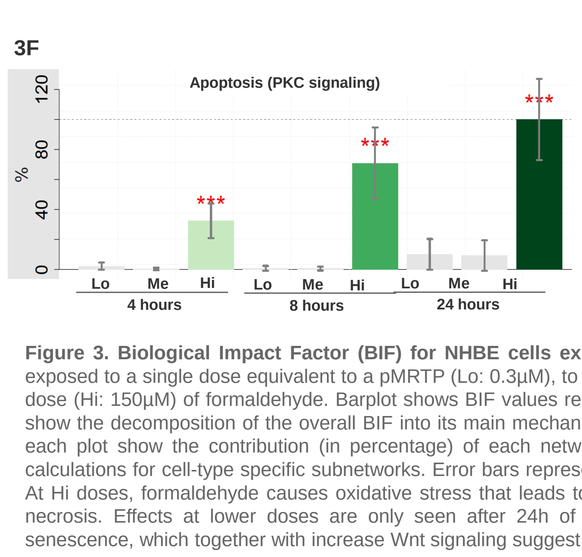


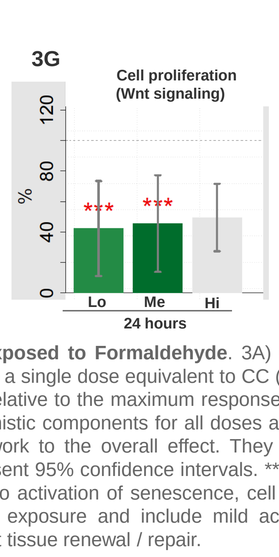












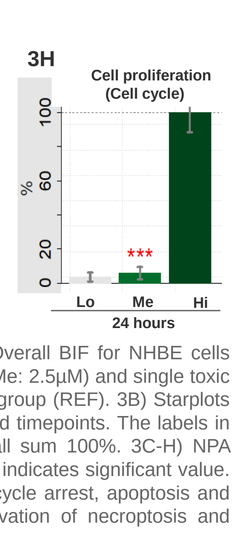


Figure 3. Biological Impact Factor (BIF) for NHBE cells exposed to Formaldehyde. 3A) Overall BIF for NHBE cells exposed to a single dose equivalent to a pMRTP (Lo: 0.3µM), to a single dose equivalent to CC (Me: 2.5µM) and single toxic dose (Hi: 150µM) of formaldehyde. Barplot shows BIF values relative to the maximum response group (REF). 3B) Starplots show the decomposition of the overall BIF into its main mechanistic components for all doses and timepoints. The labels in each plot show the contribution (in percentage) of each network to the overall effect. They all sum 100%. 3C-H) NPA calculations for cell-type specific subnetworks. Error bars represent 95% confidence intervals. *** indicates significant value. At Hi doses, formaldehyde causes oxidative stress that leads to activation of senescence, cell cycle arrest, apoptosis and necrosis. Effects at lower doses are only seen after 24h of exposure and include mild activation of necroptosis and senescence, which together with increase Wnt signaling suggest tissue renewal / repair.

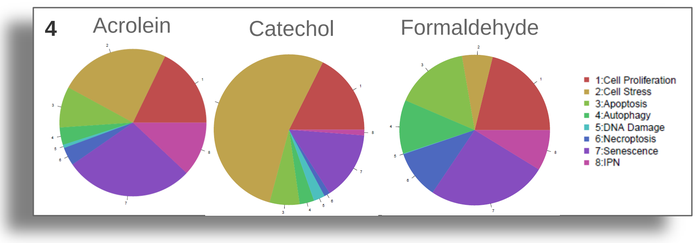


Figure 4. Starplots show the contribution of the different mechanistic components to overall toxicity of acrolein catechol and formaldehyde.

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