High Content Screening analysis of the Biological Impact of Harmful / Potentially Harmful Constituents of Tobacco Smoke

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537

INTRODUCTION

Exposure to cigarette smoke (CS) causes lung toxicity and increases the risk of developing chronic obstructive pulmonary disease and cancer [1]. CS is a complex aerosol with over 6000 chemicals. Thus, it is difficult to determine individual contributions to overall toxicity, as well as the molecular mechanisms by which smoke constituents exert their effects. Previously [2], we performed a systems toxicology evaluation of a subset of 14 CS constituents categorized as harmful/ potentially harmful constituents (HPHCs) of tobacco smoke by the U.S. Food and Drug Administration [3]. Here, we investigated the biological impact of additional 32 HPHCs using normal human bronchial epithelial (NHBE) cells. Cytotoxicity was evaluated using an impedance-based, multi-electrode array system. Additionally, 13 multi-parametric indicators of cellular toxicity were measured *via* high content screening (HCS) assays over a wide range of concentrations and at different time points (4h and 24h). Based on the HCS results, 10 HPHCs were selected for microarray-based transcriptome analysis followed by a computational approach leveraging mechanistic network models to further identify and quantify perturbed molecular pathways.

MATERIAL AND METHODS

Real-time cell analyzer

High Content Screening (HCS)

• Cell count	• Stress kinase (p-cJun)	• Mitochondrial mass
• Nuclear size	• GSH content	• Mitosis (p-H3)
• DNA structure	• Oxidative stress (ROS)	• Apoptosis (Caspase 3/7)
• DNA damage (p-H2AX)	• Mitochondrial potential	• Cell membrane permeability
		• Cytochrome C release

***Table 1*. List of HCS–based endpoints**

Network Perturbation Amplitude (NPA)

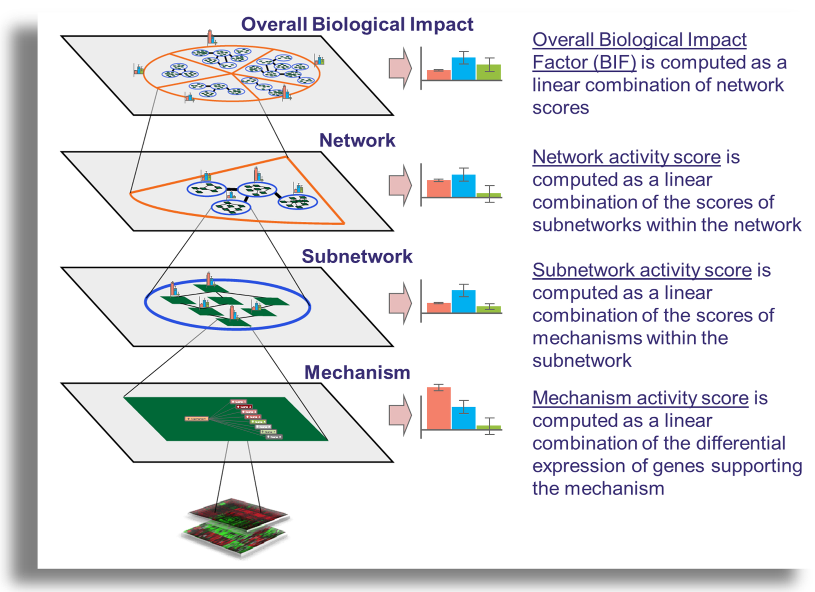

***Figure 1*. NPA scoring.** By applying the transcriptomics data to a set of network models describing key biological processes (cellular stress, apoptosis, inflammation, DNA damage, senescence, etc.), NPA scoring enables the identification and the assessment of activated molecular mechanisms in response to HPHC exposure.

Selection of HPHCs

• Arsenic (III)	• p-Cresol	• Arsenic (V)	• Acrilamide	• Benzene	• Benz [a] anthracene	• Dibenzo [a,l] pyrene
• Selenium (IV)	• m-Cresol	• Nickel (II)	• Phenol	• MEK	• Benzo [a] pyrene	• Indeno [1,2,3-cd] Pyrene
• Lead (II)	• o-Cresol	• 1-Aminonaphthalene	• 2-nitropropane	• Nitrobenzene	• Benzo [b] fluoranthene	
• Mercury (II)	• o-Anisidine	• Crotonaldehyde	• Acetamide	• Quinoline	• Benzo [k]fluoranthene	
• 5-Methylchrysene	• Naphthalene	• Chromium (VI)	• Acetone	• Toluene	• Dibenz [a,h] anthracene	

***Table 2*. List of selected HPHCs.** In blue, constituents selected for transcriptomics

CONCLUSIONS

❖ This study provides a comprehensive overview of the toxicity mechanism of a wide selection of HPHCs. While some constituents showed no toxicity in NHBE cells, HCS analysis allowed us to gain insight into the molecular mechanisms or toxicity for 17 out of 32 tested HPHCs.

❖ In a subset of 10 HPHCs, transcriptomic analysis followed by a computational approach leveraging mechanistic network models offered deeper understanding of the biological pathways impacted upon exposure. Moreover, these results from the transcriptomics analysis were in fully agreement with those from HCS.

❖ The combination of systems biology tools and high-throughput toxicity assays is a valuable approach to investigate the molecular mechanisms of toxicity. The results from this study will be used to support the approach to develop a systems biology-based risk assessment for Reduced Risk Products (RRPs).

REFERENCES

1. Rodgman, A., and Perfetti, T.A. *The Chemical Components of Tobacco and Tobacco Smoke.* 2013.
2. Gonzalez Suarez et al. Systems biology approach for evaluating the biological impact of environmental toxicants in vitro. Chem Res Toxicol. 2014.
3. *U.S. Department of Health and Human Services, Food and Drug Administration, Center for Tobacco Products. Rockville. Harmful and Potentially Harmful Constituents in Tobacco Products and Tobacco Smoke. 2012.*
4. GraphPad Prism Version 5.00 for Windows. GraphPad Software, San Diego, California, USA
5. Martin F et al. Quantification of biological network perturbations for mechanistic insight and diagnostics using two-layer causal models. BMC Bioinformatics. 2014

RESULTS

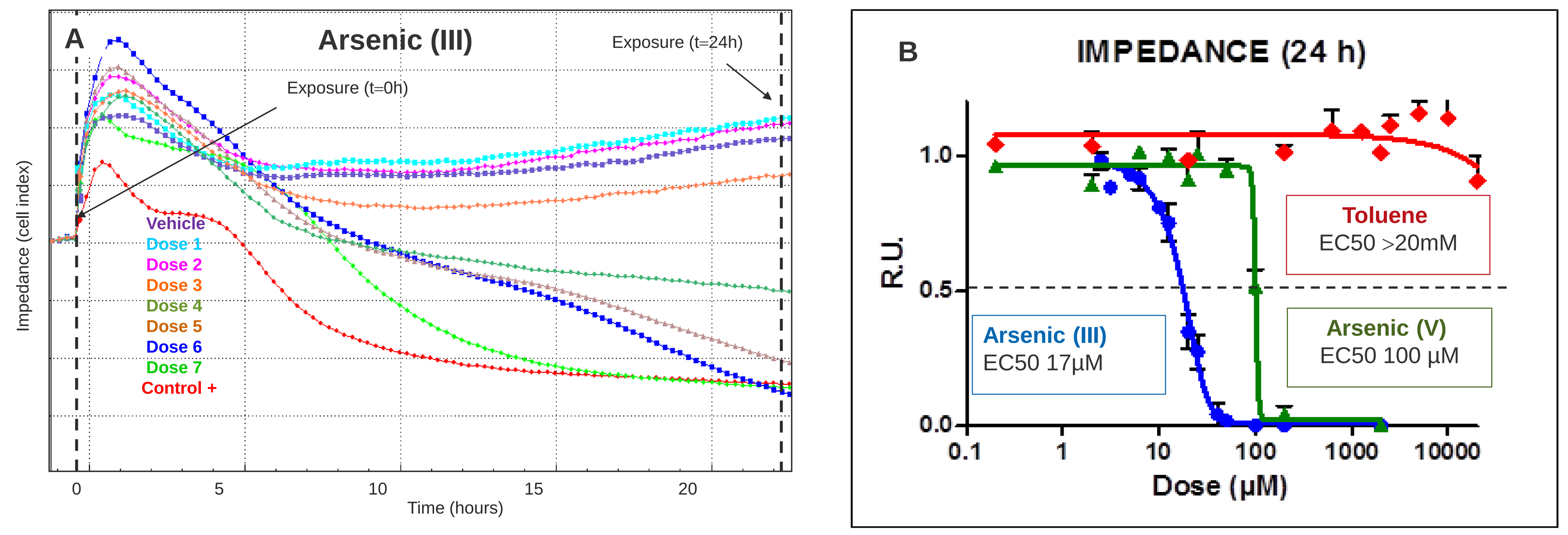

Figure 2*. Effect of HPHCs on cell viability. A)** Representative real-time cellular analysis experiment showing the response of NHBE cells to arsenic (III) (impedance is used as a readout of cell viability). Data was acquired every 15 minutes and represents the average of three replicate wells. Vertical dotted lines delimitate the exposure period (0-24h). Carbonyl cyanide m-chlorophenyl hydrazone (CCCP) was used as positive control. **B)** Impedance values at 24h post-exposure were normalized to vehicle control and fitted using GraphPad Prism® 5.0 [4] in order to calculate EC50 values. As (III), As (V) and Toluene are shown as an example. Data represents average ± SEM of at least 3 independent experiments. For all remaining HPHCs, EC50 values are shown in ***Table 3.

	HPHC	EC50 Value	R^2		HPHC	EC50 Value	R^2
1	Chromium (VI)	**4 µM**	0.995	17	o-Anisidine	**11970 µM**	0.968
2	Arsenic (III)	**17 µM**	0.968	18	2-nitropropane	**> 20 mM**	-
3	5-Methylchrysene	**28 µM**	0.961	19	Acetamide	**> 20 mM**	-
4	Arsenic (V)	**100 µM**	0.990	20	Acetone	**> 20 mM**	-
5	Mercury (II)	**110 µM**	0.999	21	Benzene	**> 20 mM**	-
6	Selenium (IV)	**338 µM**	0.982	22	MEK	**> 20 mM**	-
7	Crotonaldehyde	**501 µM**	0.994	23	Nitrobenzene	**> 20 mM**	-
8	Nickel (II)	**520 µM**	0.999	24	Quinoline	**> 20 mM**	-
9	Lead (II)	**528 µM**	0.918	25	Toluene	**> 20 mM**	-
10	1-Aminonaphthalene	**1000 µM**	0.964	26	Benz [a] anthracene	**> 100 µM**	-
11	Naphthalene	**1176 µM**	0.902	27	Benzo [a] pyrene	**> 100 µM**	-
12	m-Cresol	**2028 µM**	0.936	28	Benzo [b] fluoranthene	**> 100 µM**	-
13	o-Cresol	**2170 µM**	0.912	29	Benzo [k]fluoranthene	**> 100 µM**	-
14	p-Cresol	**5060 µM**	0.900	30	Dibenz [a,h] anthracene	**> 100 µM**	-
15	Acrilamide	**5880 µM**	0.981	31	Dibenzo [a,l] pyrene	**> 100 µM**	-
16	Phenol	**6680 µM**	0.982	32	Indeno [1,2,3-cd] Pyrene	**> 100 µM**	-

***Table 3.* EC50 values for all HPHCs.** Values were calculated at 24h of exposure using GraphPad Prism® 5.0 [4]. Only those HPHCs where an EC50 value could be calculated were further analyzed *via* HCS.

HPHC		Cell Loss	DNA Damage	Stress Kinase	GSH Content	Oxidative Stress	Caspase 3/7	Cytochrome C Release	Cell Membrane Permeability	Mitochondrial Membrane Potential	Mitochondrial Mass
5-Methylchrysene	4h	-	-	✓	✓	-	-	-	-	-	-
	24h	✓	✓	✓	✓	-	-	-	-	-	-
Arsenic (III)	4h	-	-	✓✓	✓	-	-	-	-	-	-
	24h	✓	✓	✓✓	✓✓	-	✓	✓✓	✓	✓	-
Lead (II)	4h	-	-	-	✓	-	-	-	-	✓	✓✓
	24h	✓	-	-	✓	-	-	✓	-	✓	✓✓
m-Cresol	4h	-	✓✓	✓	✓	✓	-	-	-	-	-
	24h	✓	✓✓	-	✓✓	-	✓	-	✓	✓	-
Mercury (II)	4h	-	✓✓	-	✓✓	✓	✓	✓✓	✓✓	✓✓	✓✓
	24h	✓	✓✓	-	✓✓	✓	✓✓	✓✓	✓✓	✓✓	✓✓
Naphthalene	4h	-	-	-	✓✓	✓	-	-	-	-	-
	24h	✓	✓	-	✓✓	-	-	-	-	-	-
o-Anisidine	4h	-	✓	-	✓✓	-	-	-	-	-	-
	24h	✓	✓✓	-	✓✓	-	✓✓	-	✓✓	-	✓
o-Cresol	4h	-	✓✓	-	✓✓	✓	-	-	-	-	-
	24h	✓	✓	-	✓✓	-	✓✓	✓	✓✓	-	-
p-Cresol	4h	-	✓✓	✓	✓	-	-	-	-	-	✓
	24h	✓	✓✓	✓	✓✓	-	✓	-	-	-	✓
Selenium (IV)	4h	-	✓✓	-	✓	✓	-	✓✓	-	-	✓✓
	24h	✓	✓✓	✓	✓✓	-	✓	✓✓	✓✓	-	-
1-aminonaphthalene	4h	-	✓✓	-	✓	✓✓	-	-	✓✓	-	-
	24h	✓	✓✓	✓	✓	✓✓	✓	✓	✓✓	-	-
Chromium (VI)	4h	-	✓	-	✓	-	-	-	-	-	-
	24h	-	✓✓	-	✓	-	✓✓	-	✓✓	-	-
Crotonaldehyde	4h	-	✓✓	-	-	✓✓	✓	-	✓✓	-	-
	24h	✓	✓✓	✓	-	✓	✓	✓✓	✓	✓	-
Acrylamide	4h	-	✓✓	-	✓	-	-	-	-	-	-
	24h	✓	✓✓	✓	✓	-	-	-	✓	-	-
Phenol	4h	-	✓	-	✓	-	-	-	-	-	-
	24h	✓	✓✓	-	✓	-	✓	-	✓	-	✓
Nickel (II)	4h	-	-	-	✓✓	-	-	-	-	-	-
	24h	✓	-	-	✓✓	-	✓	-	-	-	-
Arsenic (V)	4h	-	-	-	✓	-	-	-	-	-	-
	24h	✓	-	-	✓	-	-	-	-	-	-

Table 4*. Summary of HCS results.** The table summarizes the HCS results only for the 17 HPHCs with a calculated EC50 value (Table 3***). A result was considered as positive (✓✓) if a dose–dependent response was observed with at least 2–fold increase in signal over vehicle control (or a 50% decrease in signal in the case of GSH content). A signal increase between 1.5 and 2–fold (or a decrease between 30% and 50% in the case of GSH content) was considered as weakly positive (✓). Data represent results from at least 3 independent experiments. Based on the HCS results, a subset of 10 HPHCs (highlighted in blue in the table) were selected for further analysis via transcriptomics.

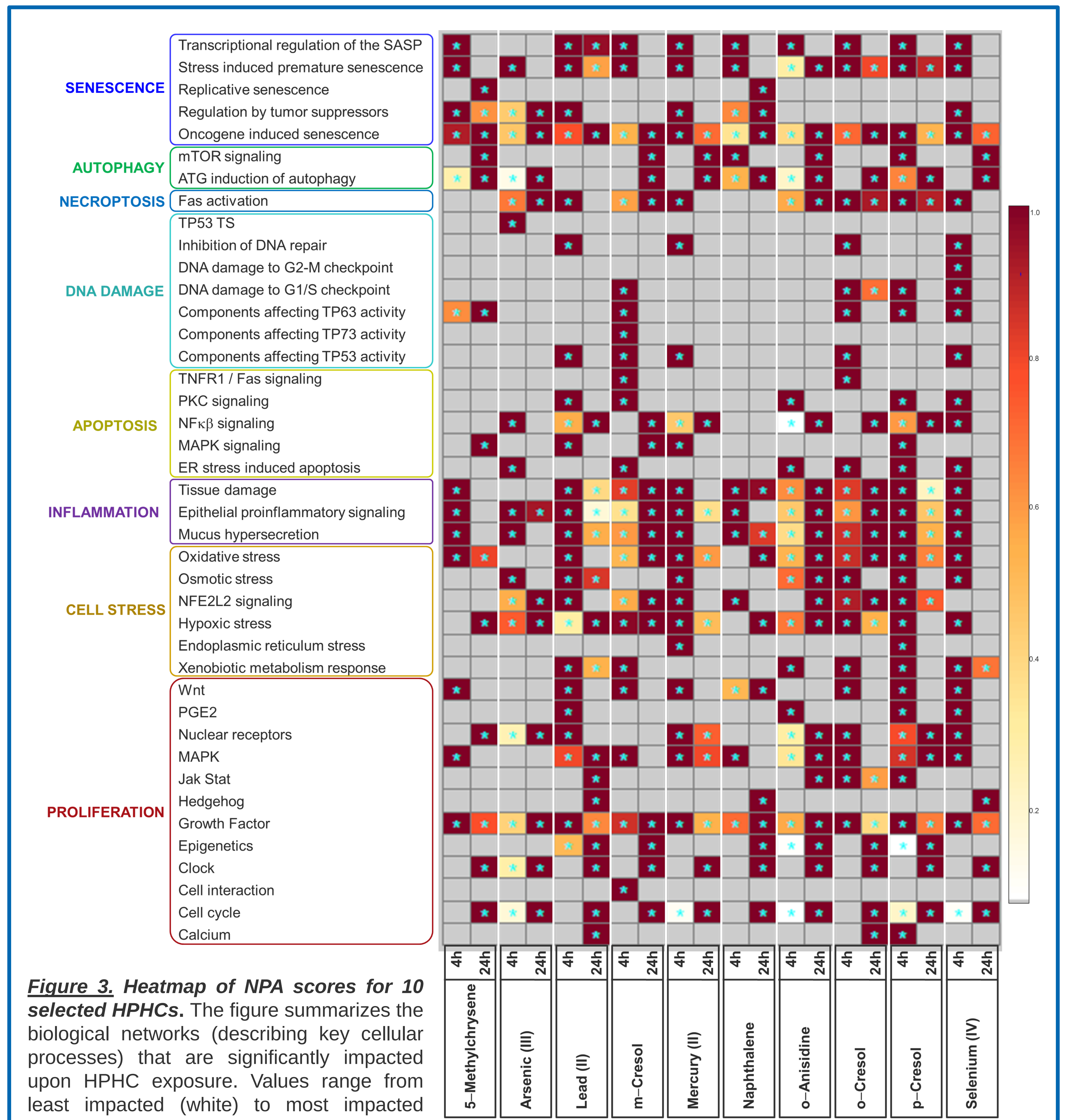

***Figure 3*. Heatmap of NPA scores for 10 selected HPHCs.** The figure summarizes the biological networks (describing key cellular processes) that are significantly impacted upon HPHC exposure. Values range from least impacted (white) to most impacted (red). NPA scores are normalized to the the most impacted biological network in each HPHC and represent the results of three independent experiments. The figure shows the results for only one dose (highest dose resulting in at least 70% cell viability at 24h) and two exposure time points (4h and 24h). ∗ indicates that NPA score is significant (p<0.05) not only with respect to the experimental variation, but also considering the biology described in the network [5]