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Biological impact of cigarette smoke compared to an aerosol produced from a prototypic modified risk tobacco product on normal human bronchial epithelial cells

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ABSTRACT

Cigarette smoking causes serious and fatal diseases. The best way for smokers to avoid health risks is to quit smoking. Using modified risk tobacco products (MRTPs) may be an alternative to reduce the harm caused for those who are unwilling to quit smoking, but little is known about the toxic effects of MRTPs, nor were the molecular mechanisms of toxicity investigated in detail.

The toxicity of an MRTP and the potential molecular mechanisms involved were investigated in high-content screening tests and whole genome transcriptomics analyses using human bronchial epithelial cells.

The prototypic (p)MRTP that was tested had less impact than reference cigarette 3R4F on the cellular oxidative stress response and cell death pathways. Higher pMRTP aerosol extract concentrations had impact on pathways associated with the detoxification of xenobiotics and the reduction of oxidative damage. A pMRTP aerosol concentration up to 18 times higher than the 3R4F caused similar perturbation effects in biological networks and led to the perturbation of networks related to cell stress, and proliferation biology.

These results may further facilitate the development of a systems toxicology-based impact assessment for use in future risk assessments in line with the 21st century toxicology paradigm, as shown here for an MRTP.

1. Introduction

Cigarette smoking causes serious and fatal diseases such as lung cancer, cardiovascular disease, and chronic obstructive pulmonary disease. Cigarette smoke (CS) is a complex mixture that contains more than 6000 identified compounds (Rodgman and Perfetti, 2013). This mixture is probably the most significant source of human exposure to toxic chemicals and the most significant cause of chemically mediated diseases in humans (Talhout et al., 2011). The World Health Organization has estimated that, globally, nearly six million premature deaths each year can be attributed to CS (World Health Organization, 2011, 2013). The long-term rates at which smokers cease smoking remain very low despite the significant efforts that have been made to control tobacco use and to communicate the risks of smoking. According to an analysis of the 2001–2010 US National Health Interview Surveys data performed by the US Centers for Disease Control and Prevention, although about 52.4% of daily smokers attempted to quit in the past year for more than a day, only 6.2% succeeded in obtaining long-term abstinence for six months or more (Centers for Disease Control and Prevention, 2011). The best way for smokers to prevent adverse health effects caused by smoking is undoubtedly to quit smoking. However, for those unable or unwilling to quit smoking, growing attention is being paid to alternative approaches, including the use of potential "reduced-risk tobacco products" (Schorp et al., 2012). The enactment of the Family Smoking Prevention and Tobacco Control Act (FSPTCA),

Abbreviations: AE, aqueous extract; CS, cigarette smoke; FC, fold change; FDA, Food and Drug Administration; fdr, false discovery rate; GSH, glutathione; HCS, high-content screening; HPHCs, harmful and potentially harmful constituents; IPA, Ingenuity Pathway Analysis; JNK, c-Jun N-terminal kinase; MRTP, modified risk tobacco product; NHBE, normal human bronchial epithelial; NPA, network perturbation amplitude; PBS, phosphate buffered saline; pMRTP, prototypic MRTP; ROS, reactive oxidative species.

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which empowers the US Food and Drug Administration (FDA) to evaluate and regulate modified risk tobacco products (MRTPs), was a significant development in tobacco control in the USA (Food and Drug Administration, 2009). An MRTP is defined in the FSPTCA as any tobacco product that is sold or distributed for use to reduce harm or the risk of tobacco-related disease associated with commercially marketed tobacco products (Food and Drug Administration, 2012b). The FDA has published a draft guidance for MRTP applications in which it is stated that such applications must provide scientific evidence to demonstrate that the product significantly reduces harm and the risk of tobacco-related disease to individual users and benefits the health of the population as a whole, taking into account both users and non-users of tobacco products. In this context, non-clinical studies are integral for evaluating MRTPs before the products can be clinically tested (Food and Drug Administration, 2012b). The prototypic MRTP (pMRTP) studied here is based on a distillation technology by which tobacco is heated rather than burned, so smaller amounts of harmful and potentially harmful constituents (HPHCs) are produced than when the tobacco is burned (Coggins et al., 1989; Food and Drug Administration, 2012a; Schorp et al., 2012; Werley et al., 2008). A fast-lighting carbon tip is used as a heat source, and the aerosol is created by heating the tobacco in a controlled manner. This generates an aerosol that is primarily composed of water, glycerol, and nicotine, and contains lower concentrations of HPHCs, such as aldehydes and polycyclic aromatic hydrocarbons, than does CS (Kogel et al., 2014).

The causal link between smoking and numerous diseases is well established (Centers for Disease Control Prevention, 2010; National Center for Chronic Disease Prevention and Health Promotion, 2014), but there is still little understanding of the underlying molecular mechanisms. *In vitro* cell culture models are important tools for studying molecular mechanisms. Multiple doses and time points can easily be tested simultaneously. The complex mixture of compounds in CS probably induces a similarly complex and multifaceted range of biological responses, and with many molecular mechanisms are likely to be involved. The biological response to CS can, therefore, be most comprehensively studied using whole genome gene expression analyses and high-content screenings, which allow this complexity to be captured. This approach is also consistent with a movement toward a new toxicity testing paradigm, a concept known as "toxicity testing in the 21st century". In this concept, it is envisaged that all toxicity testing will use human cells or cell lines in high-content settings to allow perturbations in toxicity pathways to be determined (Krewski et al., 2010; National Research Council, 2007). Toxicity pathways are cellular biology pathways that, when sufficiently perturbed, lead to adverse health outcomes (National Research Council, 2007; Stephens et al., 2012). In the "toxicity testing in the 21st century" concept, it is further suggested to move away from evaluating apical endpoints toward identifying toxicity pathways that can potentially cause adverse health effects in humans. Systems toxicology and *in silico* modeling approaches are key to improving the mechanistic understanding of toxicity pathways and integrating the data into a quantitative risk assessment framework (Bhattacharya et al., 2011; Sturla et al., 2014).

Bronchial epithelial cells, which form a first-line barrier protecting the lung from inhaled organisms and chemicals, play a major role in the pathogenesis of several CS-induced diseases. The molecular mechanisms induced by CS in lung epithelial cells were recently reviewed (Nyunoya et al., 2014). For example, CS has been found to induce inflammation, DNA damage, and autophagy in lung epithelial cells, causing the cells to undergo cellular senescence or transformation or to die through apoptosis or necrosis (Nyunoya et al., 2014). Although several studies have been conducted in which CS induced transcriptomic changes have been investigated (reviewed by Brody, 2012; Sen et al., 2007), only a few studies have been performed using lung epithelial cells. In these studies the objectives were to compare different types or brands of cigarettes, while increasing the mechanistic understanding of the effects of CS on epithelial cells. For instance, Jorgensen et al. examined the changes in gene expression in normal human bronchial epithelial (NHBE) cells exposed to CS condensates from two commercially available American brands of cigarettes over periods of up to 12 h. The majority of genes that became expressed differentially were similar for both cigarette brands. Functional pathways association analysis implicated these genes in signaling pathways affecting apoptosis, transcription, and regulation of the cell cycle (Jorgensen et al., 2004). However, each condensate also induced its own signature of genes. Pickett et al. compared the changes in gene expression after NHBE cell exposure to CS bubbled into phosphate buffered saline (PBS) preparations from 5 commercial and 4 research cigarettes whereby 21 common (at least two fold) differentially expressed genes were found, including a strongly increased expression of genes involved in xenobiotics and detoxification such as CYP1A1 and CYP1B1, and antioxidants such as GPX2 and NQO1 (Pickett et al., 2010). In addition, unique response pattern were identified for each type of cigarette. Yauk et al. exposed FE1 epithelial cells to smoke condensates from full-flavor, blonde, and "light" cigarettes, each at two concentrations for 6 h. They did not detect clear brand-specific changes in gene expression. All brands gave very similar expression patterns at each time point and concentration. The changes in gene expression were attributed to xenobiotic metabolism, oxidative stress response, DNA damage response leading to cell cycle arrest and apoptosis as well as inflammation (Yauk et al., 2012).

To date, no *in vitro* study using a whole genome transcriptomics approach describing the comparative analysis of molecular changes caused by an MRTP exposure has been published. In the study presented here, the biological effects of a pMRTP aerosol and reference CS were compared to provide additional mechanistic insights into the perturbation of cellular processes that occur when cells are exposed. NHBE cells were exposed, using different doses and two exposure periods, to an aqueous extract (AE) generated by bubbling either mainstream aerosol from the pMRTP or CS from the 3R4F reference cigarette into PBS. The examination of 11 indicators of cellular toxicity using a high-content screening (HCS) method was complemented by microarray-based whole genome transcriptomics analysis. A computational approach using pathway analysis and mechanistic network models was then used to identify the molecular pathways that had been perturbed. The aim was to facilitate the development of a systems toxicological approach for use in future risk assessment in line with the 21st century toxicology paradigm, i.e., to help to identify toxicity pathways (National Research Council, 2007) and to demonstrate the applicability of a systems toxicology approach to evaluate the risk associated with pMRTPs.

2. Materials and methods

2.1. Cigarettes, pMRTPs, and preparation of the AEs

Reference research cigarettes 3R4F were purchased from the University of Kentucky (Lexington, USA; http://www.ca.uky.edu/refcig/) and pMRTP test sticks were provided by Philip Morris Products S.A. (Neuchâtel, Switzerland). The Health Canada smoking regime (puff volume 55 mL, puff duration 2 s, puff frequency 2 min^{-1}, 100% of filter ventilation holes blocked) was used to produce the AEs. The AEs were generated by bubbling mainstream aerosol from the pMRTP or mainstream smoke from the 3R4F through ice-cold PBS in a wash bottle, thus trapping the water-soluble fraction of the aerosol or CS in the PBS. Each 3R4F reference cigarette was smoked to a butt length of 35 mm, which took between 10 and 11 puffs per cigarette, whereas a predefined puff count of 12 puffs were used for each pMRTP stick. Stock solutions containing an equivalent of 166 sticks/L (approx. 1769 puffs/L) were prepared for the 3R4F cigarettes and stock solutions containing an equivalent of 400 sticks per liter (4800 puffs/L) were