Application of Rapid *in vitro* Toxicological Screening Using ToxTracker to Determine the Effect of Flavors in Oral Tobacco Products

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Poster #99

Abstract

Modern oral tobacco nicotine products (ONP) are available in tobacco and other flavors. Traditional *in vitro* assays are not always able to differentiate toxicological impact of the different flavor profiles.

The objective of this study was to assess the mechanisms and mode of action utilizing the stem cell-based reporter assay ToxTracker to determine if different flavors of oral products can be differentiated and if they have different reporter gene induction profiles. The American style loose moist reference product (CRP 2.1) and 4 different flavored (traditional, mint, white and wintergreen) commercially available ONP were extracted with DMSO and CAS. Concentrations up to 1.6 mg/mL of the extract were tested in each of the six reporter cell lines (+/- S9) of the ToxTracker assay. Following a 24hr incubation, reporter gene (GFP) induction and cytotoxicity were assessed by flow cytometry. The induction profile for all products tested including CRP 2.1 showed a 3 to 6-fold induction of the Ddit3 (reporter for protein damage) reporter gene in the absence of S9. However, there was no reporter gene induction in the presence of metabolic activation. None of the concentrations tested had an impact on cellular cytotoxicity indicating that the doses tested were not toxic, and the concentration range could be increased.

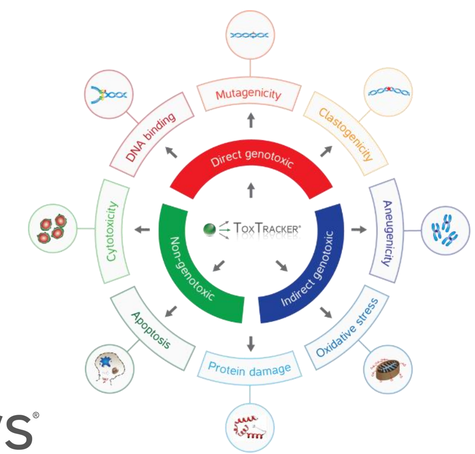
This preliminary work suggests that under the tested experimental conditions, ToxTracker is capable of distinguishing differences between flavors.

Introduction

Oral nicotine products (ONP) are available in multiple flavors ranging from wintergreen, mint, fruit, citrus and many others. The concern with flavors is two-fold: (i) it may make these products more enticing to youth and; (ii) may contribute to potential toxicity. Traditional *in vitro* toxicological assays do not detect early changes in protein damage, DNA damage, oxidative stress or cellular stress to differentiate various flavored ONP. It is possible that the flavors do not cause cellular damage that can be detected by traditional *in vitro* assays, but rather their affect may occur at the gene level by affecting specific cellular signaling pathways.

ToxTracker (Figure 1) is a stem cell-based reporter assay that provides mechanistic insight into the mode-of-action of genotoxic properties of chemicals (1, 2), may be able to differentiate between the different flavors used in ONP. The assay utilizes 6 different reporter genes that are tagged with green fluorescent protein and when induced the degree of induction can be measured via flow cytometry.

Figure 1. Different Cell Signaling Pathways in the ToxTracker Assay



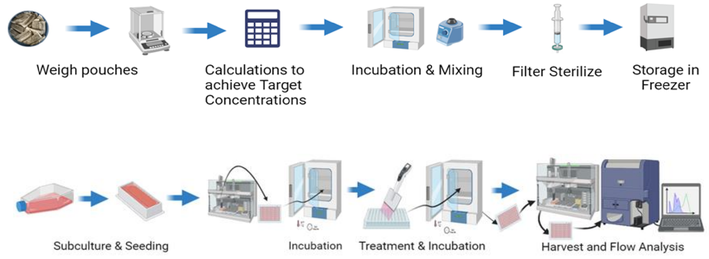
Methods

The American style loose moist reference product CRP 2.1 and different flavored (traditional, mint, white and wintergreen) commercially available modern oral products were extracted with DMSO or Complete Artificial Saliva (CAS) (Table 1). Pouches were cut open, weighed, extracted, filtered, and then stored at -20°C until assayed. Varying concentrations of the extract were applied to each of the six reporter cell lines (+/- S9) in the ToxTracker assay. Following a 24hr incubation, reporter gene (GFP) induction and cytotoxicity were assessed by flow cytometry (Figure 2). Results were normalized to the wild type (no GFP tag) cell line to account for any autofluorescence in the sample and results displayed as an increase/decrease in (GFP)-induction and relative cell survival.

Table 1.Pouch Flavors Evaluated

Solvent	Flavor	Nicotine Composition	Maximum Extract Concentration (DMSO)	Maximum Extract Concentration (CAS)
DMSO or CAS	Original	8.5 mg/g	3.0 mg/mL	6.0 mg/mL
	Wintergreen	8.5 mg/g	3.0 mg/mL	6.0 mg/mL
	Mint	8 mg/g	3.0 mg/mL	6.0 mg/mL
	White	8 mg/g	3.0 mg/mL	6.0 mg/mL

Figure 2. Sample Processing and Evaluation Procedures



Results

The CRP, original, mint, white and wintergreen flavors were extracted with DMSO. Ddit3 induction (protein damage) was observed for all the tested products (Figure 4). All of the flavored products produced a dose-dependent increase in induction of protein folding (Ddit3). Addition of S9 eliminated the induction observed with the different flavors indicating that metabolism has the potential to diminish the effect (Figure 4). There was no significant decrease in cellular survival across the concentration range tested (Figure 5).

The pouch samples were extracted with complete artificial saliva. There was no GFP-induction above the 2-fold threshold for any of the products in the presence or absence of S9 (Figures 3) nor was there any effect on cellular survival. This indicates that under CAS biorelevant extraction procedures, no response is observed and that the effect is likely not biologically relevant.

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Figure 3: Effect of ONP on (A) DNA damage, (B) Oxidative Stress and (C) Cellular Damage (-S9) with DMSO extraction; (D) CRP 2.1 cellular survival with DMSO

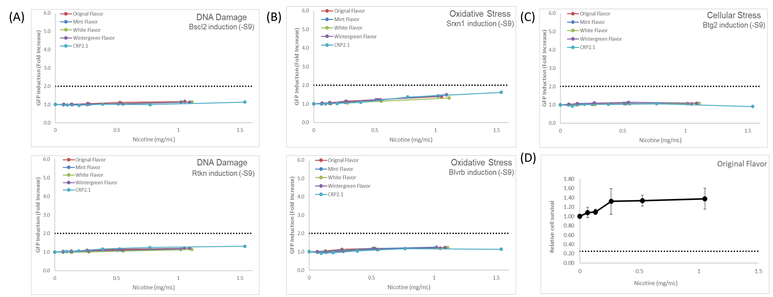


Figure 4: Effect of ONP on Protein Damage (+/-S9) with DMSO and CAS extraction

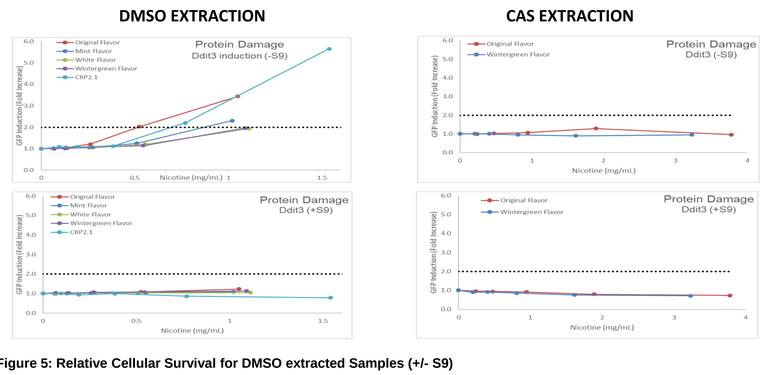
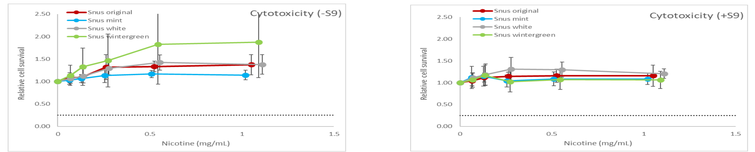


Figure 5: Relative Cellular Survival for DMSO extracted Samples (+/- S9)



Conclusions

- **Effect of Extraction Solvents:** Regardless of flavors, ToxTracker assay was able to differentiate protein damage/folding (Ddit3) observed with DMSO and absent with CAS.
- **Flavor Effect on Cytotoxicity**: There was no effect on cytotoxicity with either DMSO or CAS.
- **Flavor Effect on Oxidative Damage**: The data indicates that there was no induction of the Nrf2 pathway dependent oxidative damage-related genes (Srxn1) for any flavor.
- **Flavor Effect on Genotoxicity:** The ONPs tested did not have any effect on the induction of the AT/CHK1 DNA damage signaling pathway (Bscl2) or the NK-kB signaling pathway (Rtkn).
- **Flavor Effect on Protein Damage:** All of the flavors tested caused protein misfolding (Ddit3) suggesting that the mode-of-action is via protein damage. Ddit3 induction ranged from 3- to 6-fold for the concentration range tested.

- Additional work is still required to evaluate more flavors and to determine if the effects of flavor load.

Reference

1. Hendriks, G., Atallah, M., Morolli, B., et al. (2012). The ToxTracker Assay: Novel GFP reporter systems that provide mechanistic insight into the genotoxic properties of chemicals. Toxicological Sciences: 125(1):285-298.
2. David E. Smart, Stela Bozhilova, Fabio Miazzi, Linsey E. Haswell, Marianna D. Gaca, David Thorne, Damien Breheny, (2022). Application of ToxTracker for the toxicological assessment of tobacco and nicotine delivery products. Toxicology Letters, 358:59-68

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