Use of human derived cell lines to increase the biological relevance of cigarette condensate, non-tobacco materials and e-liquids testing

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1. INTRODUCTION

The in vitro testing strategy of tobacco products is mainly based on the testing of extracts from cigarette smoke condensates. An accepted industry wide battery of assays has been developed over recent years [1] which delivers reliable results for the toxicological assessment of tobacco products. The testing battery comprises the Neutral Red Uptake (NRU) assay, the *In Vitro* Micronucleus (IVM), and the Ames test. This combination of tests evaluates relevant biological endpoints and is also used for the toxicological evaluation of non-tobacco materials (NTM) like ingredients and e-liquids. All methods are based on the corresponding OECD guidelines which leave some choices regarding bacteria strains used in Ames test and cell lines utilized in NRU and IVM assays. With increasing experience the tests have been improved especially with regards to relevance, by using human cell lines in our biological laboratories wherever possible. The human cell based test systems have been validated in the respective tests (NRU and IVM) and representative results obtained from the exposure to total particulate matter (TPMs) and e-liquid formulations are shown. The assays lay the basis for an expanded assay battery which is under development for the assessment of next generation products (NGP).

2.1. Methods - In vitro micronucleus test (IVM)

Cell culture and IVM using TK6 / V79 cells

- V79 (ECACC; Cat.No.: 86041102) and TK6 (CLS, Cat.No.: 300357) [2,3] cells were cultivated in appropriate media supplemented with 10% serum.
- For exposures cells were seeded in 24 well plates for a 18-22 hour pre-growth phase.
- On the next day exposure solutions were added to reach target test substance concentrations without or with S9 mix (10% final concentration in well). Exposure was conducted for 3.5 hours (V79) or 4 hours (TK6). 4 increasing non-zero concentrations, one negative, and one positive control with 4 replicates each were applied per row resulting in 6 different exposure groups per 24 well plate.
- After 4 hours the cells were washed (V79) or re-suspended in fresh medium (TK6) (10% Serum) to allow for the expression of micronuclei (MN) (2). Cells were then centrifuged to microscopic slides and stained with 4'6'-Diamidin-2-Phenylindol (DAPI).
- MN evaluation was done using the Metafer4 software coupled to a fully automated microscope (Imager Z2, Zeiss).
- Due to higher variability for TK6 MN frequencies 4 counts per dose are performed, while for V79 cell preparations 2 counts are sufficient. For the same reason TK6 cells are used rather to assess the genotoxicity in a yes/no based manner while comparison of combustible products is done with V79 hamster lung fibroblasts (**Fig.1** and **Fig.2**).

Qualitative data using TK6 cells

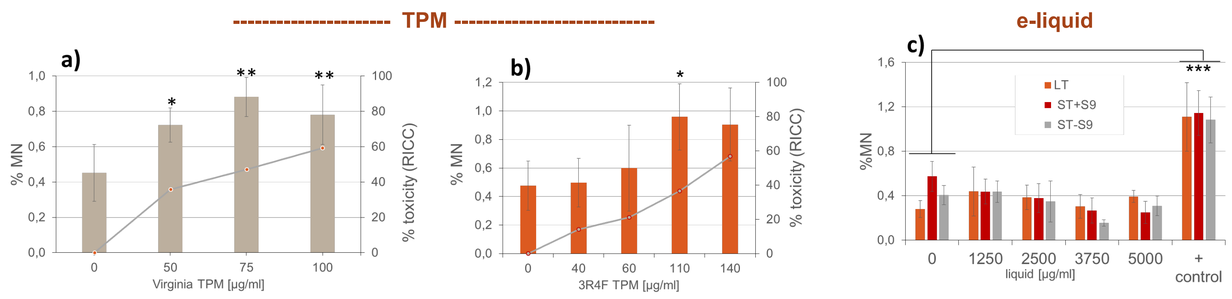

Fig.1: TK6 cells were exposed to TPM from Virginia bend test cigarettes **(a)** and 3R4F reference cigarettes **(b)** in the presence of S9. Statistically significant increases as compare to the negative control are indicated by asterisks (ANOVA with $p<0.05$). Columns indicate the genotoxicity and lines describe the cytotoxicity associated. **(c)** shows representative results of a qualitative analysis of an e-liquid formulation with regard to its genotoxic potential. The data indicate that e-liquid has no genotoxic properties; appropriate positive controls induced statistically significant increases in MN frequencies (not shown for TPM experiments).

Quantitative data using V79 cells

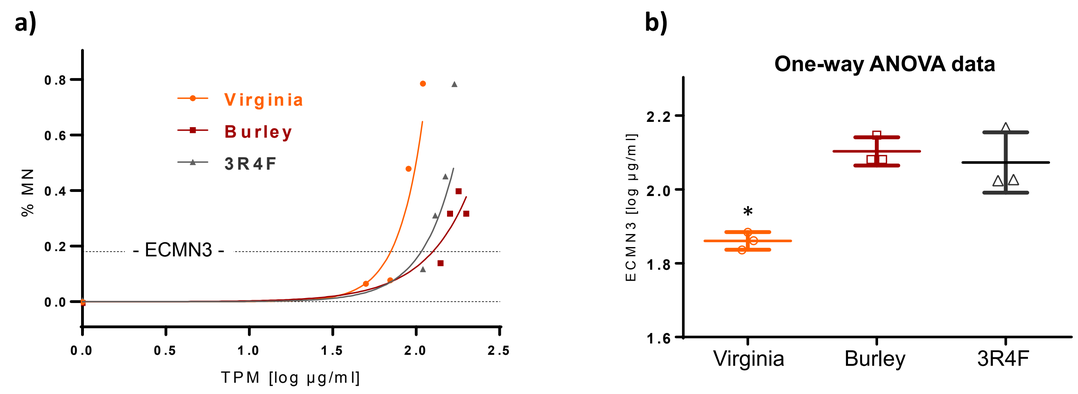

Fig.2: **(a)** The dose dependent increase of micronucleus containing V79 cells after exposure to TPM from Virginia, Burley and 3R4F combustible test articles. From the dose response curves a non-linear regression was used for the calculation of the dose that induced a threefold increase of MN frequencies over background levels (ECMN3). **(b)** shows the statistical comparison of ECMN3 values (One- way ANOVA) as the measure for genotoxicity. Data basis: results from 3 independent test days per TPM type.

2.2. Methods - Neutral Red Uptake assay (NRU)

Cell culture and Neutral Red Uptake assay (HepG2 and Beas-2B cells)

- BEAS-2B (ECACC; Cat.No.: 95102433) bronchial cells were grown in bronchial epithelial growth medium (BEGM; Lonza). HepG2 (ATCC, Cat.No.: HB8065) liver cells were grown in MEM & Weymouth's 705/1 (4:1) supplemented with 1% Ultroser-G.
- Both cell lines cells were seeded into 96 well plates to optimized cell densities which allow constant growth over the whole exposure period.
- On the next day the 4-fold concentrated TPM exposure solution was added to a final volume of 200µl medium per well to achieve the target condensate level. For NTM testing the supernatant is completely replaced by 200µl exposure solution.
- After exposure (65 ± 4 hours) neutral red staining / extraction was applied. From the absorption values (540nm) EC50 determination was conducted using GraphPad Prism 6.07 statistical software **(Fig.3)**.

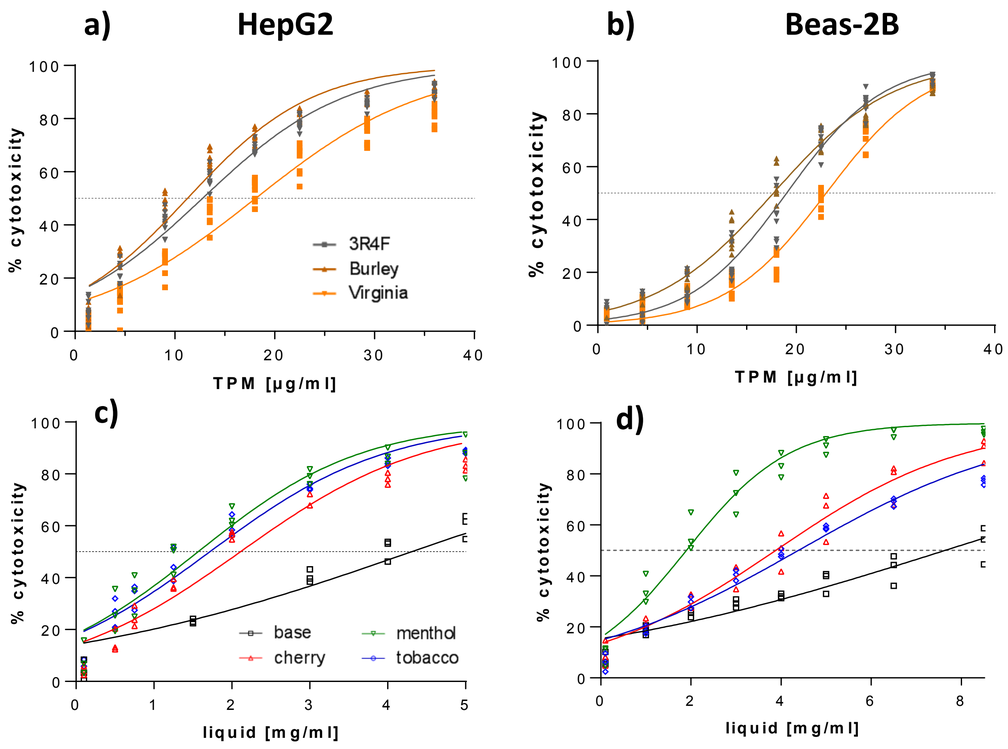

Fig.3:Toxicity profile obtained after exposure of **(a)** HepG2 and **(b)** BEAS-2B cells to increasing concentrations of 3 different TPM extracts from Virginia blend test cigarettes, Burley test cigarettes and standard reference cigarettes (3R4F) combustible test articles. Figures **(c)** and **(d)** show the toxicity profile of representative e-liquid formulations used to expose HepG2 cells and Beas-2B cells respectively.

2.3. Methods - Ames test

- The *Salmonella typhimurium* reverse mutation assay (Ames test) was conducted according to the OECD guideline #471 [4].
- With TPM from combustible products strains TA98, TA100, in the presence of S9 show best dose response characteristics (see **Fig.4**).
- To determine the mutagenic potential of NTMs/e-liquids the strains TA1535, TA1537, and TA102 were used according to OECD guideline #471 (data not shown).

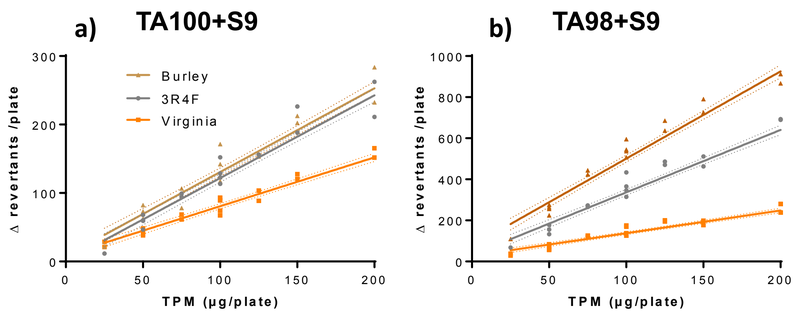

Fig.4:Figures describing the dose dependent increase of revertants after exposure of TA100 **(a)** and TA98 **(b)** to TPM from different test articles in the presence of S9 mix.

3. CONCLUSIONS

The data indicate the suitability of the presented human derived test systems for the testing of combustible tobacco products and NTMs.
The TK6 cells used in the IVM show higher variability than cells from Chinese hamster lung V79. Therefore TK6 cells are used rather for qualitative evaluations with e-liquids or NTMs.
Combustible products and NGP based test matrices (e-liquid) induce highly reproducible dose response profiles when using human derived cell lines in the NRU test.
The classically used Ames strains are suited for the determination of mutagenic potential of NTMs and samples from combustible products.
The presented assay battery will be expanded to also cover endpoints related to cardio vascular disease and chronic obstructive pulmonary disease.
For the assessment strategy for fresh smoke / aerosol please refer to poster; Biological Test procedure for fresh generated smoke and aerosols (STPOST 52)

REFERENCES

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