ST 2 - Roper - *In vitro* cytotoxicity of cigarette mainstream smoke. Evaluation of different cell exposure methods ...

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In vitro cytotoxicity of cigarette mainstream smoke. Evaluation of different cell exposure methods, including 'native' smoke aerosol exposure.

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Summary

The determination of cytotoxicity, e.g. by Neutral Red Uptake (NRU) viability or MTS metabolic activity assays, is an essential part of an *in vitro* mammalian cell culture test battery for cigarette smoke (CS). However, appropriate testing agents (i.e. smoke fractions vs. whole smoke) need to be identified, in order to expose the cells to as many toxicologically relevant smoke constitutents as possible. Generally, cells suspended in liquid medium may be exposed to CS condensate ('tar') generated by standardized procedures and dissolved in appropriate solvents like DMSO (dimethyl sulphoxide). As an alternative, cells may be treated with aliquots of aequous extracts of CS gas phase or total CS, generated by 'bubbling' gas phase or whole CS through cell culture medium. We have evaluated the cytotoxicity of smoke of cigarettes with different filter types by NRU and/or MTS assays using human HEP-G2 liver hepatoma or A-549 lung carcinoma cells and the micro titre plate (MTP) technique. Cells were exposed to DMSO solutions of 'tar' as well as aequous extracts of gas phase and whole CS. Indeed, exposure of a thin layer of cells ('air-liquid' culture) to the native CS aerosol rather than 'tar' or smoke extracts would be more realistic and similar to exposure of lung cells of a smoker. Thus, a single-port smoking machine was developed, enabling us to expose cells cultured in MTPs to variable amounts of freshly generated CS aerosol. Data determined after exposing cells to smoke indicate a different cytotoxicity (evaluated by cigarette equivalent) of different smoke fractions. The effects of the tested principal filter designs on cytotoxicity could clearly be demonstrated, in the course of which A-549 showed similar ranking of test cigarettes but less sensitivity to cigarette smoke. As a new technique, CS aerosol exposure is feasible and may be a tool for the future.

Introduction

The evaluation of toxicological effects of tobacco smoke is gaining importance, partly, because regulatory authorities may require these data to evaluate e.g. the use of certain tobacco additives or the concept of so-called 'potential reduced exposure products'. In vitro toxicity testing of tobacco smoke is a fundamental part of an appropriate test battery, which may include tests for bacterial mutagenicity, mammalian in vitro genotoxicity and mammalian in vitro cytotoxicity. When setting up test systems for cigarette smoke, the unique properties of smoke as a complex aerosol have to be considered, i.e. effects of both

particulate and vapour phase should be taken into account. We here report data of cytotoxicity tests with mammalian cell cultures using two different cell lines and different fractions of cigarette smoke. Six experimental cigarettes of different filter design (acetate, paper, carbon, ventilated and non-ventilated) were used as test pieces.

Materials and Methods

Experimental cigarettes

Six experimental cigarettes of the American Blend type according to Table 1 were used throughout the study:

Table 1: Experimental cigarettes

Code	Filter	Specifications	Filter vent. [%]	ISO-'Tar' [mg/cig]	ISO-Nic [mg/cig]
1001	acetate, 21 mm	2.1Y/36000SK	0	10.9	0.8
2002	acetate, 21 mm	2.1Y/36000SK	30	8.4	0.7
3003	acetate, 21 mm	2.1Y/36000SK	60	4.4	0.4
4004	none	PE-tube, 21 mm, with tipping	0	22.7	1.4
5005	paper, 21 mm	35 g/m^2	0	9.0	0.5
6006	ac-carbon-ac, 9/5/7 mm	chamber with 80 mg carbon BR123	0	10.3	0.7

Cell lines

Cell lines employed included

- HEP-G2 (human liver hepatoma, ATCC HB-8065), cultured in serum-free medium.
- A-549 (human lung carcinoma, DSMZ ACC 107), cultured in serum-free medium.

Smoke exposure

Cells were exposed to smoke condensate, aequous extracts of vapour phase and whole smoke, as well as freshly generated whole smoke aerosol. This was done in 96-well MTP's, containing 2×10^4 cells per well in serum-free medium, pre-cultured for 22 hours in this environment.

- Condensate was collected on 92 mm Cambridge filter pads by smoking 20 cigarettes according to the ISO standard. Filters were extracted with 20 ml DMSO. Aliquots of this stock solution were diluted, as appropriate, added to the wells to give seven to eight final condensate concentrations ranging from 2 to 92 µg/ml, depending on cell line and assay used, and incubated 65 hours at 37 °C.
- Vapour phase or whole smoke extracts were prepared using a single port smoking machine (35 ml puff, 2 seconds, puff interval 58 seconds). Smoke of three cigarettes was consecutively bubbled through a washing flask containing 15 ml of serum-free culture medium. Aliquots of this extract were added to the culture wells (eight concentrations) and cells were incubated as described above.

- Native smoke aerosol exposure of cell monolayers cultured in MTP's was done using the device shown in Figure 1. Its main part is a single port smoking machine linked to an exposure chamber with a glass cover (volume ca. 1.7 L). One MTP is placed at the bottom of the chamber; wells contain a minimum amount (10 µl) of culture medium. A motor-controlled stainless steel sliding lid may be used to cover and release wells, thus exposing rows of wells to different numbers of puffs. Directly after puffing, the puff is blown into the chamber. Electronic valve and piston speed control allow various inter-puff dilution and air-flushing regimes, thus mimicking human smoking and breathing behaviour. Before incubating cells for 65 hours at 37 °C after smoke exposure, fresh nutrient medium (190 µl) was added to each well.

Fig. 1: Whole cigarette smoke aerosol cell exposure apparatus

Cytotoxicity assays

Neutral Red Uptake (NRU) and MTS assays were performed according to references (1), (2), and (3), respectively. Cytotoxicity data are shown as dose-response curves, cytotoxicity vs. dose, compared to untreated control wells. Data refer to cytotoxicity per mg of condensate and cytotoxicity per cigarette equivalent, respectively. From these curves, smoke doses for 20%, 50%, and 80% cytotoxicity (EC_{20}, EC_{50}, EC_{80}) were calculated.

Results

Condensates and smoke extracts

As an example, EC_{50} data of the HEP-G2 cell line are given in Table 2. Typical NRU and MTS dose response curves are shown in figures 2 and 3, respectively. For condensate on a per mg basis, we found a somewhat higher toxicity of the samples with filter ventilation. For

smoke extracts on a per cigarette basis, samples with filter ventilation and the sample with a carbon filter clearly exhibit less toxicity.

Table 2: EC_{50} data for HEP-G2 cells
Condensate and extracts of vapour phase/whole smoke

code	condensate [µg/ml]		vapour phase [cig./ml]		whole smoke [cig./ml]	
	NRU	MTS	NRU	MTS	NRU	MTS
1001	16.5	48.2	0.027	0.015	0.007	0.009
2002	11.7	44.2	0.039	0.026	0.009	0.013
3003	11.9	41.9	0.100 (EC_{40})	0.097	0.021	0.030
4004	17	60.1	0.021	0.016	0.002	0.003
5005	16.4	56.1	0.025	0.016	0.006	0.007
6006	15.7	53.5	0.054	0.038	0.010	0.013

NRU vs. MTS assay

Data of both assays are compared in Figures 4 and 5. As for condensate, NRU seems to be more sensitive than MTS; as for the vapour phase extract, MTS is more sensitive than NRU.

HEP-G2 vs. A-549 cell line

A-549 cells exhibited a far less sensitive response to smoke condensates and whole smoke extracts (vapour phase not tested in both cell lines), which may in part be due to the different metabolic competence of the two cell lines. With regard to whole smoke extracts, the effective dose of smoke extracts had to be roughly two (MTS) to four times (NRU) that used for the HEP-G2 cells to gain similar effects with A-549 (Fig. 6). For condensates, doses for A-549 cells had to be two- to five-fold (data not shown).

Condensate vs. smoke extracts

To directly compare the toxicity data of condensate and smoke extracts we expressed the condensate dose (originally given as µg/ml) as cigarette equivalents per ml. Data of condensates, vapour phase, and whole smoke extracts are now shown in one graph (Figures 7 and 8). The figures illustrate that, for a given cigarette on a per cigarette basis, condensate is far more toxic than vapour phase extract, whole smoke extract has an intermediate cytotoxicity.

Whole smoke aerosol exposure

HEP-G2 cell exposure to smoke aerosol was done by consecutively smoking three cigarettes (8 puffs) of each test piece using the device shown in Figure 1. Mainstreams smoke generation and cell exposure in the chamber was controlled using the following parameters:

Puff volume was 35 mL, puff duration two seconds, and puff interval 58 seconds, the number of puffs drawn was eight. Immediately after puffing, the smoke aerosol was exhausted within 0.5 seconds into the exposure chamber. During the puff interval nine three-second flushing cycles of the chamber with 150 mL ambient air were done before the next puff was taken

from the cigarette. Our data indicate that - given these airflow conditions - cells tolerated exposure to up to five "sham smoked" (unlit) cigarettes without damage.

To control for different smoke doses in one experiment, eight rows of MTP-wells were exposed to different numbers of puffs (3 to 24 puffs) using a sliding lid releasing an additional row of wells before each puff. Thus, the smoke dose for each row of wells (more correctly, the smoke dose in the exposure chamber, when the row was released and not covered by the lid) was: 1st row: all eight puffs of three cigarettes (24 puffs), 2nd row: puff two to eight (21 puffs), 3rd row: puff three to eight (18 puffs), 4th row: puff four to eight (15 puffs), etc, and final row: last puff of three cigarettes (3 puffs). The NRU cytotoxicity data are presented in figure 9, showing clear dose-effect curves. As can be seen from the graph, it is possible to distinguish cytotoxic effects of the test cigarettes (5005>1001>6006>2002>>3003), the ultra low tar cigarette (3003) giving no toxic response under these experimental conditions. In addition, data gathered so far indicate as well that the toxic effects we observe after whole smoke aerosol exposure in this systems are due to **both** particulate and vapour phase effects; indeed, vapour phase only exposure gave a far less pronounced toxic effect compared to whole smoke exposure (data not shown).

Conclusion

From our data we conclude that with the methods that have been described we are able to distinguish the cytotoxicity of experimental cigarettes with different filter designs. Depending on the objectives of cytotoxicity studies with cigarettes, tests with smoke condensate, vapour phase or whole smoke aerosol allow a reasonable 'ranking' of cigarettes with regard to their cytotoxicity. These tests may be part of a future test battery for the evaluation of modified products.

References

1. Borenfreund E., Puerner J.A. (1985): Toxicity determined in vitro by morphological alterations and neutral red absorption. Toxicol Lett **24,** 119-124.
2. Riss TL, Moravec, RA (1992): Comparison of MTT, XTT, and a novel tetrazolium compound MTS for in vitro proliferation and chemosensitivity assays. Mol Biol Cell **3**, Suppl 184a
3. Promega Technical Bulletin No. 169 (http://www.shpromega.com.cn/tb169.pdf)

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Figures

Fig.2: Cytotoxicity of condensates, dose-response curves (NRU assay)

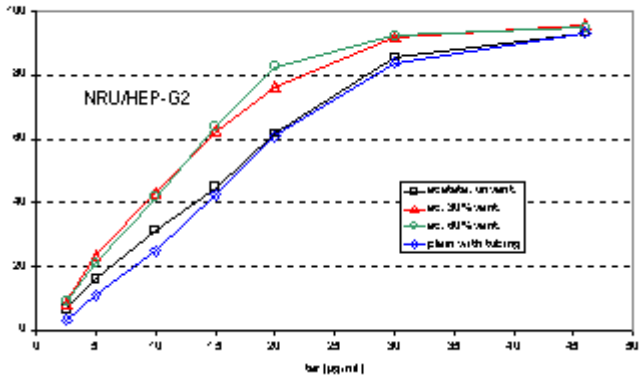

Fig.3: Cytotoxicity of condensates, dose-response curves (MTS assay)

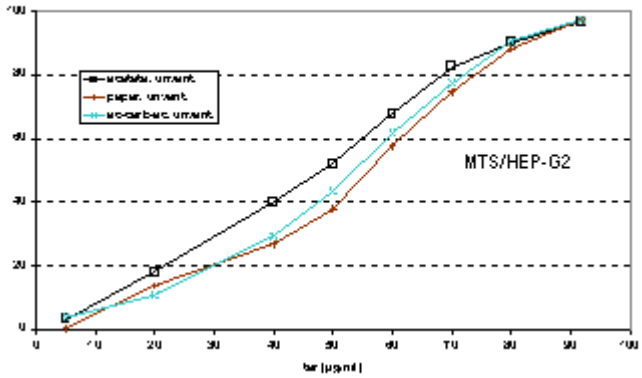

Fig.4: Cytotoxicity (EC50) of condensates, NRU vs. MTS assay (HEP-G2)

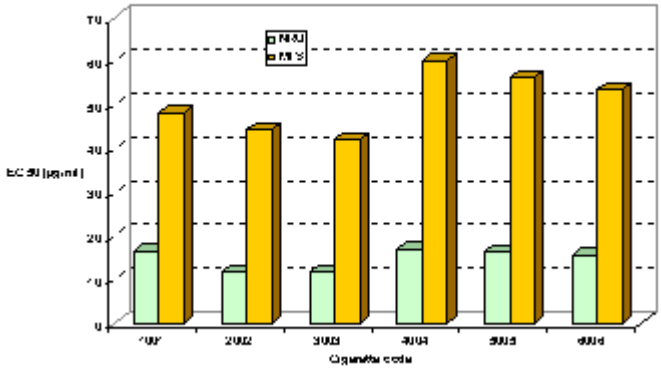

Fig.5 : Cytotoxicity (EC50) of vapour phase, NRU vs. MTS assay (HEP-G2)

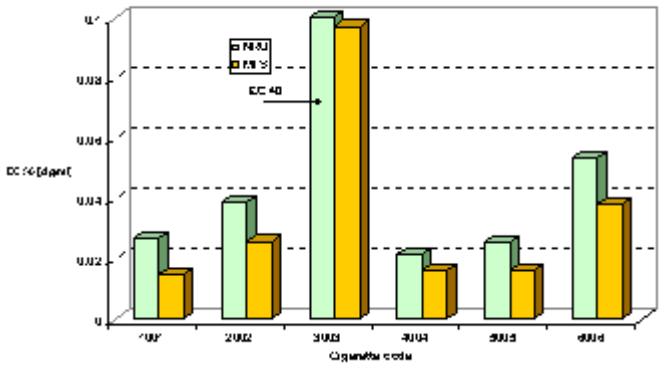

Fig.6 : NRU Cytotoxicity (EC 50) of tobacco whole smoke extract, A-549 vs. HEPG2 cells

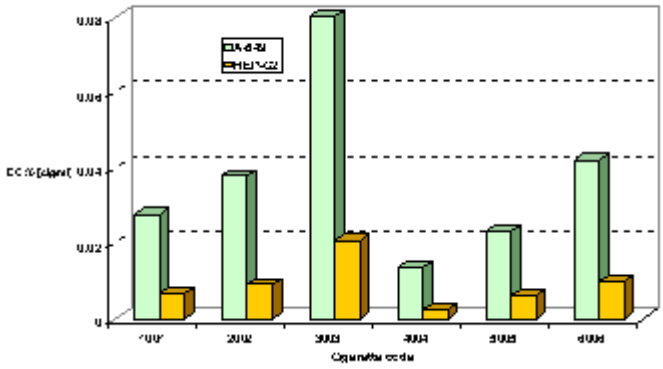

Fig.7 : Cytotoxicity of different test compounds, cigarette 1001

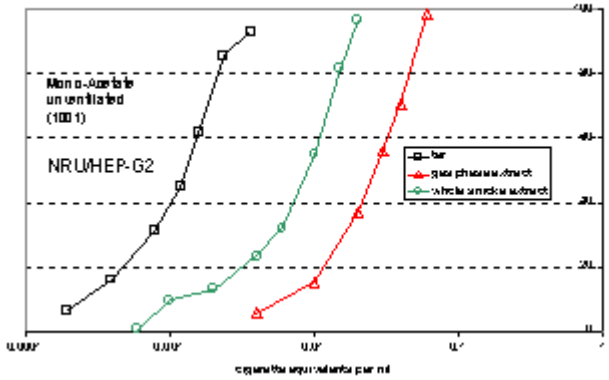

Fig.8: Cytotoxicity of different test compounds, cigarette 6006

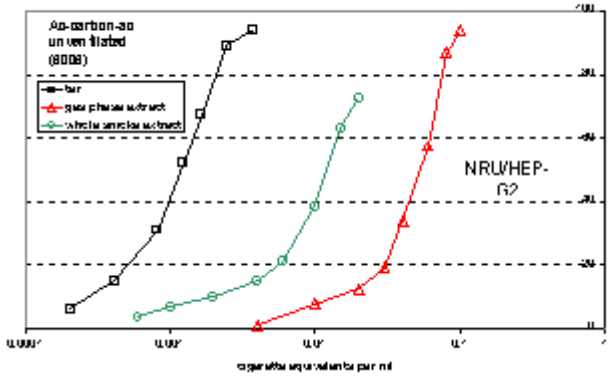

Fig. 9: Cytotoxicity of cigarette smoke aerosol (HEP-G2, NRU assay)