ST 19 - Röper - Strategies to assess the biological properties of tobacco smoke

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Strategies to assess the biological properties of tobacco smoke

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Abstract

In addition to measuring tobacco smoke analytes, there will be an increasing demand in the future to establish guidelines for testing the biological activity of tobacco smoke. Thus, testing methods have been identified which can provide valid data in terms of the cytotoxic and genotoxic potential of tobacco smoke. As a prerequisite, the tests performed in our laboratory included a battery of *in vitro* short term tests rather than animal experiments. In addition, our test results allow a quantitative evaluation in order to be able to compare different products. With regard to some biological effects of tobacco smoke known from the literature, an appropriate testing battery consisted of the following: The Ames microbial mutagenicity assay with TA 98 to assess genotoxicity of tobacco smoke condensate, a ciliate motility assay (with *Tetrahymena vorax*) to assess ciliatoxicity of the gaseous phase or whole tobacco mainstream smoke, and the neutral red cytotoxicity assay using HEP-G2 human cell line. Besides that som other effects on mammalian cell cultures were evaluated by commercially available micro titer plate (MTP) assay kits. Some testing data of cigarette mainstream smoke will be presented to serve as examples for the routine application of these methods, including description of factors influencing the outcome of these tests.

Biological effects of tobacco smoke

The assessment of biological effects of tobacco smoke or tobacco smoking *in vivo* and *in vitro* has been published in numerous studies over the past decades, including epidemiological studies, mouse skin painting and animal inhalation experiments, as well as studies using *in vitro* cell culture systems.

While animal experiments are not only time consuming (and quite costly), but also unacceptable due to ethical considerations and/or legal requirements in a number of countries, *in vitro* systems might be a future tool to evaluate the biological activity of tobacco smoke, as required *e.g.* by product development.

These testing systems ("test battery") may also at least in part be useful in compiling toxicological data with regard to ingredients used in the manufacture of tobacco products.

According to scientific literature quite a number of diseases have been associated with tobacco smoking. These so-called "smoking-related diseases" include *inter alia* cancer diseases (*e.g.* lung cancer), cardiovascular heart diseases (CHD) or chronic obstructive lung diseases (COLD). Thus, a meaningful test battery should include testing systems being sensitive to at least some of the factors (tobacco smoke constituents) held responsible for the

initiation and progress of the named diseases, *e.g.* according to the simplified Table 1:

Table 1. Diseases, risk factors, and assumed biological endpoints

Disease	factors	Biological endpoints
Cancer	initiators free radicals promoters	genotoxicity clastogenicity cell transformation
CHD	carbon monoxide nicotine free radicals oxidants	lipid peroxidation red./ox. Glutathione
COLD	NOx free radicals cytotoxic agents ciliotoxic agents	cytotoxicity elastase/antielastase balance "lung clearance"

Addtionally, the cell systems to be chosen should:

- be applicable to the full spectrum of cigarettes available in the market (*i.e.* from plain to ultra low tar),
- yield results that allow quantification of the observed effect to be able to compare the data of different cigarettes,
- yield data that allow statistical evaluation,
- allow automation wherever possible, *e.g.* use micro titer plate (MTP) systems for mammalian cell cultures.

Test battery employed

To meet requirements, biological testing systems used in the Reemtsma laboratories have continuously been developed and improved, and are under constant review.

At present, systems include as a standard:

- AMES microbial mutagenicity assay with strain TA 98 (*Salmonella typhimurium*) and S9 metabolic activation (cf. ref. 3),
- ciliastasis test with the ciliate *Tetrahymena vorax* (Protozoa, Ciliatae), currently adapted following the procedure of Gräf *et al.* (cf. ref. 2),
- cytotoxicity test with *Tetrahymena vorax*,
- Neutral Red uptake (NRU) toxicity test with human (liver) cell line HEP-G2 (cf. ref. 1),
- MTS cell proliferation test with HEP-G2 (cf. ref. 4), using MTS tetrazolium compound (Owen's reagent)

Some details of testing procedures are given in Table 2.

Table 2. Biotest procedures

Test	cs smoke generation, collection*	smoke application	endpoint measur
AMES	ISO, RM-20, condensate collected on CF**	condensate in DMSO	revertants per mg condensate
Ciliastasis (*Tetrahymena*)	RM-1, gas phase	small exposure chamber, 5 µl of culture exposed	complete ciliastasis (seconds from start o exposure)
Cytotoxicity (*Tetrahymena*)	RM-1, mainstream smoke or gas phase collected in aqueous solution	aliquots of "smoke extract" added to nutrient medium, inoculum of cells added	cell proliferation (cell density after 24 hours incubation)
NRU	ISO, RM-20, condensate collected on CF RM-1, mainstream smoke or gas phase collected in aqueous solution	condensate in DMSO aliquots of "smoke extract" added to culture	cell vitality, neutral re uptake
MTS	ISO, RM-20, condensate collected on CF RM-1, mainstream smoke or gas phase collected in aqueous solution	condensate in DMSO aliquots of "smoke extract" added to culture	cell proliferation (as measured by intensity formazan dye)

*CS: cigarette smoke** CF: Cambridge filter

The collected data are then evaluated according to Table 3.

Table 3. Data evaluation

Test	data generated	data calculated	data reported
AMES	dose-response curve (condensate per plate vs. revertants minus background)	revertants induced by e.g. 100 µg of smoke condensate, revertants per cigarette	relative mutagenicity mg condensate/per cigarette (sample vs. comparison)
Ciliastasis (*Tetrahymena*)	seconds until complete ciliastasis		seconds until comple ciliastasis
Cytotoxicity (*Tetrahymena*)	dose-response curve ("puffs" added to culture vs. cell density)	"puff aliquots" inducing 50% growth retardation compared to untreated control	relative cytotoxicity p puff/per cigarette (sar vs. comparison)
NRU	dose-response curve ("smoke" per well vs. cytotoxicity compared to untreated controls)	dose inducing a cytotoxicity of 20%, 50%, and 80%, resp., compared to control	relative dose of smok induce 20% (50%, 80 cytotoxicity (sample v comparison)
MTS	same as NRU	same as NRU	same as NRU

Examples

Use of the methods described above aims *inter alia* at:

- identifying fundamental factors that may increase or decrease biological activity, such as tobacco type, cigarette design, filter design etc.
- describing the effects of cigarette ingredients on biological activity
- evaluating our own products to be put on the market as well as competitors' products

Four examples are given in the figures 1 to 4, which show some of the results obtained with the Ames assay, the *Tetrahymena* ciliastasis and cytotoxicity assay, and the mammalian MTS cell culture assay. These include the effects of tobacco type (dark vs. bright), charcoal filters, and tobacco ingredients such as casing and flavour. Finally, table 4 very briefly lists a few of the factors influencing the outcome of testing.

- **Mutagenicity (*Salmonella typhimurium* TA 98, + S9)**

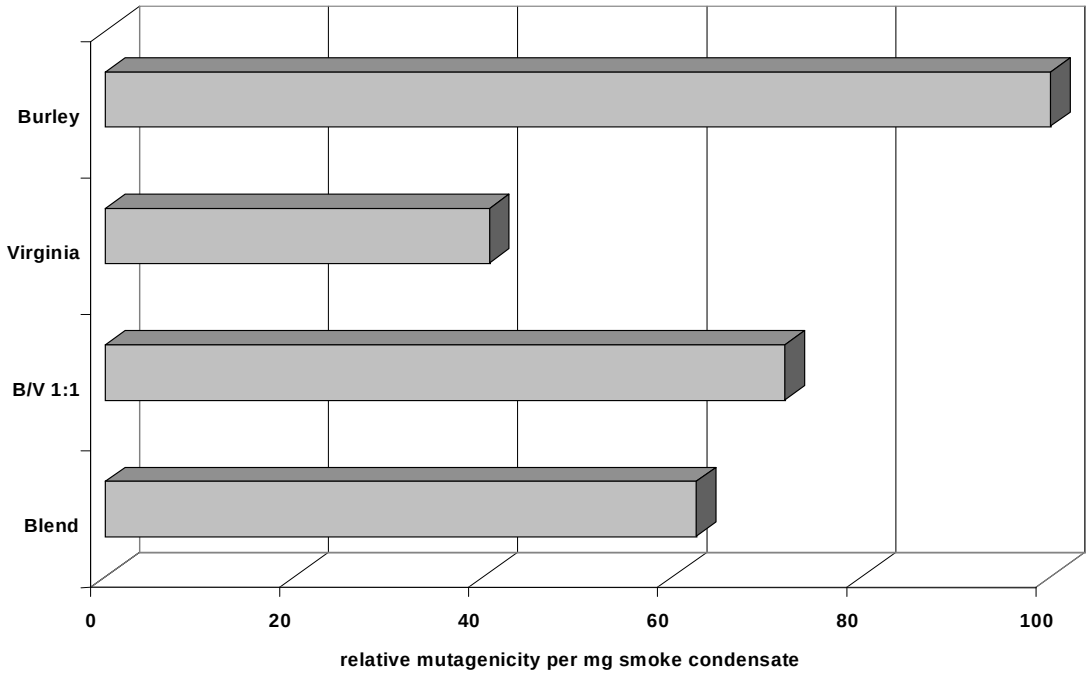

Fig. 1 - Relative mutagenicity of smoke condensates of different tobacco types

- **Cytotoxicity (*Tetrahymena vorax*, Ciliatae)**

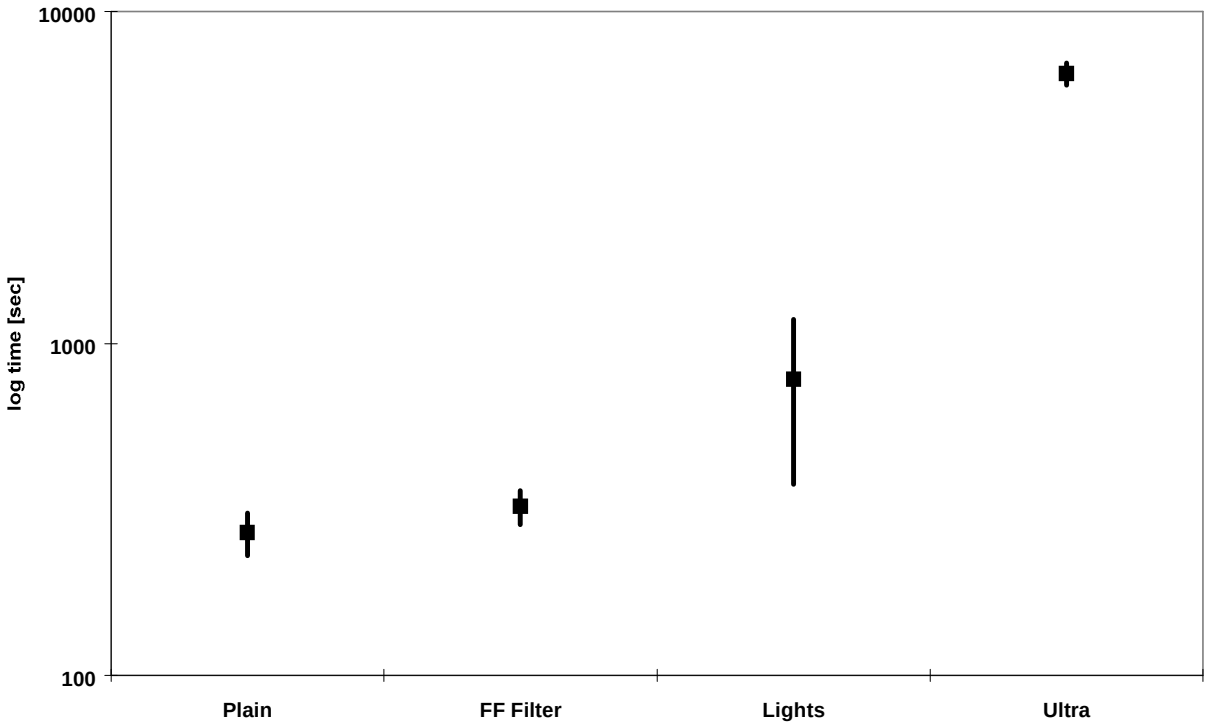

Fig. 2 - Ciliastasis of *Tetrahymena* [log seconds] by exposure to gas phase of different cigarettes
(mean values; bar indicates range found for different products); FF Full Flavour

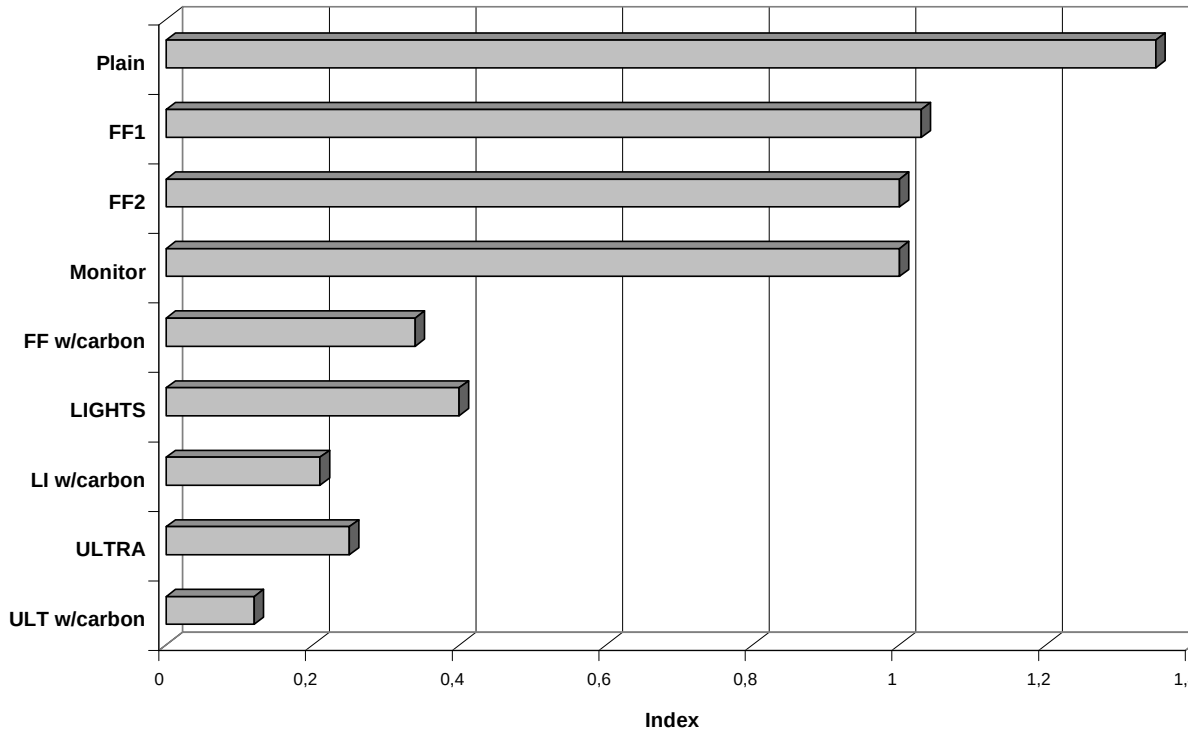

Fig. 3 - Relative cytotoxicity of cigarette mainstream smoke, including plain, full flavour

(FF), light and ultra light cigarettes. and the effects of charcoal filters, as measured with *Tetrahymena vorax*

- **Cytotoxicity (mammalian cell culture)**

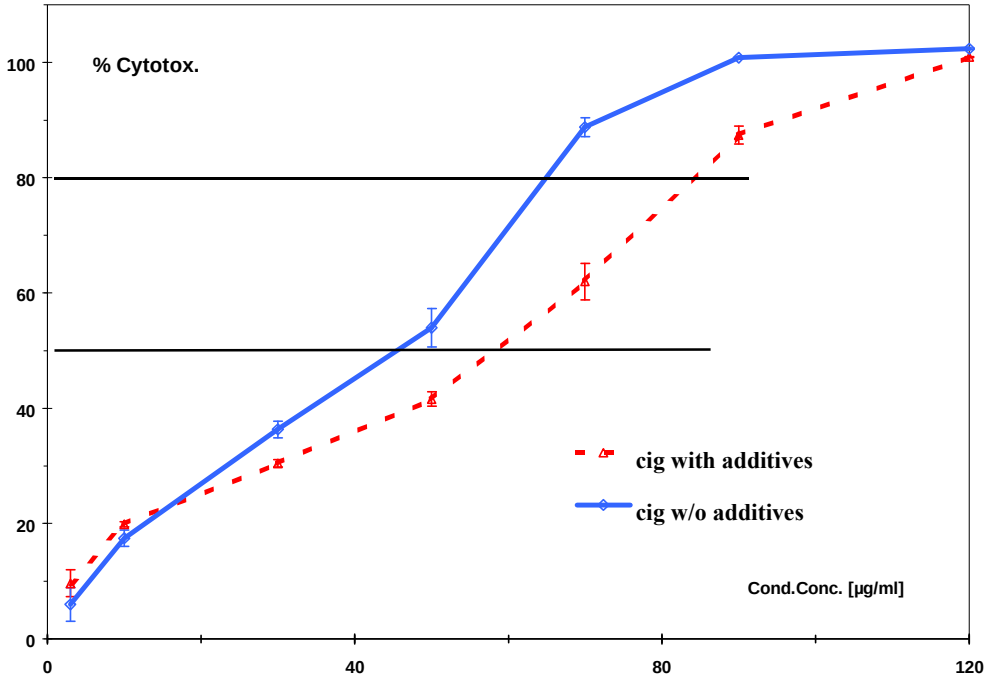

Fig. 4 - Effects of tobacco additives on the cytotoxicity of mainstream smoke condensate, as measured with human HEP-G2 cell line (MTS cell proliferation test)

Table 4 - A few cigarette design factors influencing experimental results

Endpoint	factor	effect
Bacterial mutagenicity (TA 98 + S9)	cigarette "tar"	rather good correlation between "tar" and cigarette mutagenicity
	tobacco type	"specific" (per mg) mutagenicity Burley>American Blend>Viriginia
	charcoal filter	no effect
	tobacco additives	no effect
Cytotoxicity (gas phase)	filter ventilation	inverse correlation
	charcoal filter	significant reduction
	tobacco type	dark > bright
Cytotoxicity (whole mainstream smoke / condensate)	tobacco additives	depending on amount added; no effect or less toxic

Outlook

The test battery outlined above covers but a few aspects of *in vitro* toxicology. Thus, more research will be done to enlarge the battery. We are going to evaluate and adopt additional test systems, in order to improve the validity (in terms of predicative power) and total quality of our results.

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. Gräf W. *et al.* (1998/99): *Tetrahymena pyriformis* in the ciliate mobility test. Validation and description of a testing procedure for the registration of harmful substances in the air as well as the effects of cigarette smoke on the human respiratory ciliated epithelium. Zent bl Hyg Umweltmed **201**, 451-472.
3. Maron DM, Ames BN (1983): Revised methods for the *Salmonella* mutagenicity test. Mutat Res **113**, 173-215
4. 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