The resolving power of *in vitro* genotoxicity assays for cigarette smoke particulate matter

K Scott[1], I Crooks[1], J Saul[2], O M Camacho[1], D M Dillon[1], C Meredith[1]

[1]British American Tobacco, Group Research & Development, Regents Park Road, Southampton, SO15 8TL, UK
[2]Covance Laboratories Ltd., Otley Road, Harrogate, North Yorkshire, HG3 1PY, UK

Corresponding author: ian_crooks@bat.com

INTRODUCTION

•Cigarette smoke particulate matter (PM) is genotoxic *in vitro* and novel tobacco materials can reduce PM genotoxicity (1, 2)
•Accurate estimation of the reduction of genotoxicity requires quantitative interpretation of the results
•The aim of this poster is to recommend (i) statistical methods for the quantitative interpretation of the Ames test, *in vitro* mouse lymphoma assay (MLA) and *in vitro* micronucleus test (IVMNT), (ii) the number of replicate cultures per dose, when comparing cigarette smoke PMs

METHODS

Test article preparation

•3R4F reference cigarettes were conditioned according to ISO 3402:1999
•Cigarettes were smoked on a RM200 smoking machine using ISO standard puffing parameters (3) & 300mg of cigarette PM collected on 44mm Cambridge filter pads
•PM was then eluted in DMSO to a concentration of 24mg/ml

Statistical methods

•Transformed data were tested for linearity and significance as shown in Fig. 1
•Significant differences were identified using analysis of covariance (ANCOVA) (slope and magnitude) or t-tests (common doses). Prior to the t-test, Ames and IVMNT data were rank transformed

In vitro assays

The *in vitro* assays were performed as described in (2), with the following exceptions:
I.Ames test: Only 3 strains were used (TA98, TA100 and TA1537) in the presence of S9, with 8 replicate plates per dose
II.MLA: 6 replicate cultures per dose were exposed for 24 h without S9
III.IVMNT: 6 replicate V79 cell cultures per dose were pulsed with test article for 3 h followed by a 21 h recovery without S9
IV.The above represent the most sensitive treatment conditions and the initial replication levels were selected following a review of historical data, indicating the scope to increase resolving power and to evaluate the statistical methods

RESULTS

Estimation of replication level

•Power calculations were performed on the slopes of the dose responses, pooled data and each concentration separately, to estimate the number of replicates per concentration that would detect a 30% increase or decrease in the response, with 80% power, at p<0.05. The results are summarised in Table 1.
•The levels of replication typically used in these assays (e.g. 3 in the Ames test, 2 in MLA and IVMNT), could resolve a 30% difference in PM genotoxicity, in terms of slope.
•Replication levels of 5 (Ames test TA98), 4 (Ames test TA 100), 10 (Ames test TA1537), 6 (MLA) and 3 (IVMNT) would be required for similar resolution, in terms of pooled data or individual doses.

Table 1. Estimation of replication levels to resolve different PMs in the Ames test, MLA and IVMNT.

Assay◊		Replicate cultures			Resolvable difference (%)+		
		Slope	Pooled	Dose	Slope	Pooled	Dose
Ames test*	TA98	1 - 10	5	5	10.8 - 11.9	30	30
	TA100	1 - 10	3	4	17.8 - 19.6	30	30
	TA1537#	1 - 10	10	8	23.8 - 26.2	30	30
MLAº		1 - 10	6	6	23.8 - 26.2	30	30
IVMNT^		2 - 10	3	3	28.2 - 29.9	30	30

◊ Data generated using the replication levels given in Materials and Methods; + 80% power, p<0.05, two-tailed, compared to 3R4F PM; * with S9; #outliers removed; o 24 hour treatment without S9; ^ 3 hour treatment without S9.

Confirmation of replication levels

•Two 3R4F PMs (from the same PM stock solution, but one sample was diluted to 70% (v/v), to simulate a 30% difference between PMs) were compared.
•Replication levels were as described in Table 1 for comparisons at common doses, except for IVMNT where 4 replicate cultures per dose were used, if no linearity was observed.
•Linearity was identified in all assays (Table 2). Differences between the PM samples were statistically significant in all three assays.
•This confirmed that replication levels of 5 (Ames test TA98), 4 (Ames test TA 100), 10 (Ames test TA1537), 6 (MLA)* and 4 (IVMNT) can resolve 30% differences in PM genotoxicity.

Table 2. Confirmation of the resolving power of the Ames test, MLA and IVMNT for different PMs

Assay		Slope of the dose response+	
		3R4F PM	70% (v/v) 3R4F PM
Ames test	TA98	1.353	0.891*
	TA100	0.357	0.285*
	TA1537	0.088	0.059*º
MLA		0.0258	0.0182*
IVMNT#		0.0374	0.0300*

+ revertants/µg PM (Ames test), log MF/µg PM (MLA), proportion of MnBn cells/µg PM; * significantly lower than 3R4F PM, p<0.01 (Ames test, IVMNT), p<0.05 (MLA); o outliers excluded; # slopes were equal, so compared as mean responses.

*In the MLA, six replicate cultures per dose might restrict experimental design by exceeding the maximum number of cultures in one study. In this case, reducing the replication level to four cultures per dose would have a negligible effect on resolving power (30-40%).

Statistical Methods

Figure 1 demonstrates the statistical methods to compare PM mutagenicity and genotoxicity in the Ames test, MLA and IVMNT.

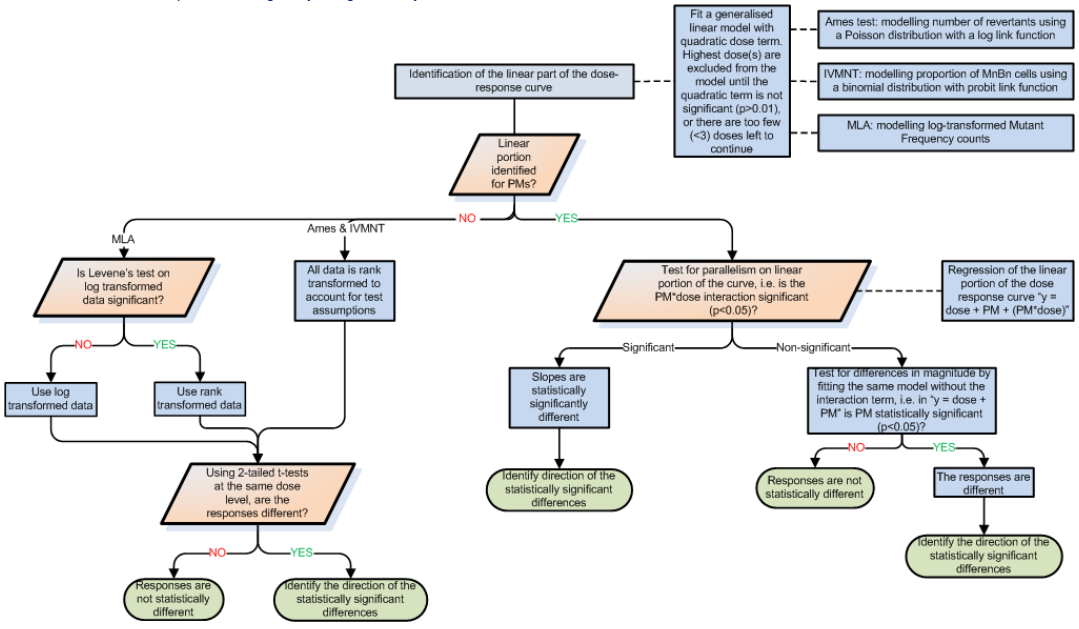

Figure 1. A common statistical approach for the evaluation of mutagenicity and genotoxicity data when comparing different PMs.

CONCLUSIONS

1. A common statistical approach was developed, for the comparison of different PMs, in the Ames test, MLA and IVMNT.
2. Using appropriate levels of replication, the Ames test, MLA and IVMNT resolved a 30% difference in PM genotoxicity.

REFERENCES

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